DIFFERENTIATING MONOCYTES INTO NEURONS

by

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ABSTRACT

This thesis addresses the development of an *in vitro* model system that will allow researchers to study the development and function of neurons from people with neurological disorders. Future studies predicated on the development of this *in vitro* system will help define how neurons malfunction, thus facilitating therapeutic development and possibly a cure for certain neurological diseases.

The first goal of this thesis was to determine if terminally differentiated blood monocytes harvested from human peripheral blood could be reprogrammed into neurons. To accomplish this conversion, human monocytes were de-differentiated to reach a pluripotent state, termed induced pluripotent stem (iPS) cells. These iPS cells have many of the same characteristics as human embryonic stem (hES) cells, including immortality and pluripotency. Previous work has demonstrated that terminally differentiated primary human skin fibroblasts have been de-differentiated into iPS cells. The expression of four transcription factors, Oct-4, Sox-2, Klf-4 and c-Myc, is essential for de-differentiation of primary cells, such as monocytes from humans. Forced expression of these transcription factors can be accomplished by using retroviral vectors. iPS cells are then isolated, expanded, and characterized to ensure immortality and pluripotency. Subsequently these pluripotent cells are directed by different growth factors to differentiate into neurons.

The results presented in this thesis demonstrate the differentiation of the H9 line of hES cells into neurons. To confirm that H9 cells were converted into neurons, gene

and protein expression levels of stem cell and neuron markers were determined by real-time polymerase chain reaction (PCR), microarray and flow cytometry, respectively. Furthermore, iPS cells have been created from mouse fibroblasts using a lentivirus vector thereby demonstrating the efficacy of this system.

In conclusion, this thesis will aid in the derivation of iPS cells from blood monocytes and their subsequent differentiation into neurons. Future studies will be directed towards terminally differentiating iPS cells into neurons that could potentially be used to study developmental, genetic and phenotypic abnormalities of cells from patients with neurological disorders.

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LIST OF ABBREVIATIONS

Induced pluripotent stem (iPS)
Human embryonic stem (hES)
Polymerase chain reaction (PCR)
Peripheral blood mononuclear cell (PBMC)
Embryonic body (EB)
Human neural progenitors (hNP)
Leukemic inhibitory factor (LIF)
Bovine fibroblast growth factor (bFGF)

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INTRODUCTION

A subset of cells harvested from human preimplantation embryos possess characteristics that allow them to expand, proliferate and differentiate into any different cell type *in vivo*, as well as *in vitro*. This property makes these hES cells extremely valuable as a research tool because it allows for the study of the development and function of many different cell types. In addition, hES cells are potentially valuable for therapeutic use because they could be implanted into patients where they would develop into cells, tissues and organs that would repair body parts that have been injured by disease or trauma. However, the use of hES cells is laden with ethical concerns because of the sensitive nature of the material from which the cells are obtained. An embryo must be destroyed to harvest hES cells, and many people feel that the destruction of human embryos is never justified, regardless of the benefits that could result from research using the material. These individuals feel that the destruction of an embryo is similar to abortion and equates to killing a human being. This viewpoint persists even though it is possible to use embryos that were created by *in vitro* fertilization and would be discarded otherwise, or to use embryos that have been donated to research specifically for this purpose (Green, 2002).

Currently the number of hES cell lines available for distribution is limited (http://www.wicell.org/index.php?option=com_oscommerce&Itemid=192), and each of these cell lines has additional limitations regarding availability, accessibility, expense,

and intellectual property rights. Also, federal funding for research on hES cells is a persistent problem in the United States, and any cell lines used in research with federal funding must meet stringent guidelines regarding how the original cells were obtained. It is possible to obtain stem cells from human adult tissues such as bone marrow and umbilical cord, but these stem cells are multipotent, not pluripotent, and only have the ability to differentiate into cell lineage types specific for the tissue from which they were obtained (Markowicz, 2008).

Because of the aforementioned difficulties in working with hES cells, researchers have sought methods to de-differentiate terminally differentiated somatic cells to create immortal, induced pluripotent cells (iPS cells), that possess the same characteristics as hES cells. One can then direct the differentiation of these iPS cells *in vitro* into various and specific somatic cell types, including neurons, effectively reprogramming the cells.

BACKGROUND

Embryonic Stem Cells

History

The first pluripotent mammalian embryonic stem (ES) cells to be characterized were discovered in the mouse blastocyst and embryo and were selected because they possess characteristics that included the ability to initiate the formation of chimeric animals and to form teratocarcinomas (Evans and Kaufman, 1981, Martin, 1981). These cells were shown to be pluripotent by the formation of a wide variety of morphologically distinct cell types including giant cells, cells resembling neurons, endodermal cells, cartilage cells and kidney tubules (Martin, 1981). Also, cells were characterized by their expression of cell surface antigens and through analysis of cellular proteins by gel electrophoresis (Evans and Kaufman, 1981). After ES cell lines were established and characterized in the rodent, the first nonhuman primate ES cell line was created in 1995 using Rhesus monkey embryos by Dr. James A. Thomson (Thomson et al., 1995). These cells were also selected because of their ability to form teratocarcinomas. Specific surface antigens of primate ES cells were described in this paper. Many of these are the same antigens that appear on mouse cells.

The first stable hES cell lines to be created in the lab were reported by Dr. Thomson in 1998 in the journal *Science* (Thomson et al., 1998). These cell lines are distinct from pluripotent teratocarcinoma-derived cells as determined by cell surface

makers, high levels of telomerase activity and a normal karyotype. The cells were taken from embryos produced by *in vitro* fertilization that were donated by patients from a medical center in Israel. They were cultured until they reached the blastocyst stage and the inner cell masses were removed. These original isolates include the H1, H7, H9, H13 and H14 lines, which are still being used by researchers today. In Thomson et al. (1998) true pluripotent stem cells are defined as those that are able to proliferate indefinitely without differentiating (immortal), and that possess the potential to differentiate into the three embryonic germ layers, which include endoderm, mesoderm and ectoderm.

Characteristics

Confirmed hES cell lines are able to form benign teratocarcinomas upon transplantation into immunodeficient mouse recipients. Teratocarcinomas are germ cell tumors composed of differentiated somatic tissues (Blum et al., 2009). One method of confirming continuous proliferation and pluripotency of cells is to inject them into severe combined immunodeficient (SCID) mice or SCID-beige mice (Amit et al., 2000, Reubinoff et. al., 2000). SCID mice are not immunocompetent and are unable to suppress uncontrolled proliferation of cells, while beige mice are unable to form lysosomes, which compromises innate immune function. The formation of all three germ layers in the resulting teratomas indicates that cells are able to undergo differentiation. It is important to note that hES cells are different from embryonic carcinoma (EC) cells, in that hES cells are derived from preimplantation embryos, whereas EC cells are undifferentiated cells contained in a teratocarcinoma (Thomson and Odorico, 2000). Interestingly, it has also been shown that cells called embryonic germ (EG) cells derived from embryos at later stages than the blastocyst also exhibit pluripotency and may prove

to be a useful resource in the characterization and manipulation of pluripotent stem cells (Thomson and Odorico, 2000).

hES cells express cell surface markers including stage-specific embryonic antigen (SSEA)-3, SSEA-4, TRA-1-60, TRA-1-81 (the TRA antigens are named after the Battle of Trafalgar; TRA does not stand for "tumor rejection antigen" as is believed by some) and alkaline phosphatase, that distinguish them from terminally differentiated cells (Thomson et al., 1995). hES cells also express the transcription factor octamer motif ATGCAAAT-4 (Oct-4), SRY related HMG Box gene 2 (Sox-2), kruppel-like factor 4 (Klf-4) and myelocytomatosis viral oncogene homolog (c-Myc) (Reubinoff et al., 2000). Once cells lose the ability to differentiate into various cell types, they also lose the expression of these markers and exhibit an increased expression of SSEA-1 (Thomson et al., 1995).

It has been hypothesized that telomere length is an indication of cell longevity, such that telomere shortening is an indication of cell senescence. Also, it has been observed that activity of the enzyme telomerase is essential for maintenance of telomere length in nonmalignant cells. Preservation of telomere length and high telomerase activity is observed in germ cells and tumor cells where proliferative capacity is high, as well as in ES cells (Amit et al, 2000). In fact, telomerase activity persists in ES cell lines that have undergone more than 300 population doublings, which is more than four times the life span of a normal somatic cell, indicating immortality (Thomson et al., 1998, Amit et al., 2000). Telomerase activity can be detected by a method called the telomerase repeat amplification protocol (TRAP) where the telomerase reaction product is detected by the polymerase chain reaction (PCR) (Savoysky et al., 1996).

The karyotype of hES cells is normal, in that chromosomal number and morphology is the same as differentiated somatic cells. The first three lines isolated from male cells (H1, H13 and H14) have a normal XY karyotype, while the first two lines isolated from female cells (H7 and H9) have a normal XX karyotype (Thomson et al., 1998). It is important that hES cells maintain a normal diploid karyotype throughout their unlimited expansion. This further distinguishes them from teratocarcinoma cells, which have aberrant chromosomal structures and are aneuploid (Thomson et al., 1995).

Isolation

In 1998, Thomson et al. used a method to isolate hES cells that had previously been used to isolate nonhuman primate stem cells (Thomson et al., 1995). Variations on the method are still widely used today in isolating and maintaining cultures of hES cells (Lerou et al., 2008, Park et al., 2008a). Embryos are cultured to the blastocyst stage (70-100 cells) in G1.2 and G2.2 tissue culture medium and the inner cell masses are isolated by immunosurgery. In immunosurgery, the embryo is incubated in acidic tyrodes solution (a solution with high osmolarity) to digest the zona pelludica and expose the trophectoderm cells. Then the structure is incubated with a rabbit anti-human red blood cell primary antibody and guinea-pig complement serum to lyse the trophectoderm cells, which are subsequently removed by pipetting up and down in a glass pipette (Chen and Melton, 2007).

The isolated inner mass cells are plated on feeder cells (irradiated mouse embryonic fibroblasts, or MEFs) and grown in supplemented Dulbecco's Modified Eagle Medium (DMEM) for 9-15 days. Outgrowths are then dissociated into clumps and re-plated on fresh feeder cells. Growth is observed until isolated colony formation occurs,

after which colonies are then individually selected and expanded. At this point cells can be frozen and stored for future use (Thomson et al., 1998).

Feeder Cells

hES cells are unable to survive and proliferate in culture unless they are supported by a layer of feeder cells or some other type of substrate. Feeder cells are usually composed of inactivated MEFs (Reubinoff et al., 2000). The feeder cells are inactivated so that they will not divide. They release necessary nutrients into the medium, including leukemic inhibitory factor (LIF) and bone morphogenic proteins (BMPs) which maintain pluripotency. Feeder cells also provide a sticky surface on the culture dish so that cells from the inner cell mass can attach. Without this layer of feeder cells the hES cells will undergo differentiation and cease proliferating. Also, hES cells are unable to grow efficiently without serum in the culture medium (Amit et al., 2000). Unfortunately, the presence of serum in the culture medium increases the potential for hES cells to begin differentiating, so substitutions are often used, such as Knockout Serum Replacement (Invitrogen).

The requirements for a feeder layer and serum in the culture medium decrease the potential for therapeutic use in humans because of concerns regarding contamination with animal products. In 2001 a method was described for successfully culturing hES cells without a layer of feeder cells, instead using a substrate called Matrigel (Xu et al., 2001). Matrigel is composed of laminin, collagen IV and heparin sulfate. In Xu et al. (2001), conditioned MEF medium was used as the growth medium for ES cells. Recently a method was described (Ludwig et al., 2006) that allows the culture of hES cells on a cell support matrix consisting of human derived collagen IV, fibronectin, laminin, and

vitronectin, also eliminating the need for a feeder layer. In addition, the cells were cultured in serum free medium called mTeSR that contains all of the necessary components for growth, plus the addition of high levels of bovine fibroblast growth factor (bFGF), transforming growth factor β (TGFβ), gamma-aminobutyric acid (GABA), pipecolic acid and lithium chloride. These defined conditions allow for high reproducibility and limited variation. Also, since there are no feeder cells or serum from animals, the potential is greatly increased for cells grown in this manner to be used therapeutically.

ES cells that are grown on a feeder layer of MEFs grow in colonies that have a distinctive morphology. The colonies have well-defined borders; they are round, flat, and are encircled by a bright halo. This is different from ES cells that are grown on Matrigel in mTeSR medium. These colonies also have well-defined borders, but they are relatively polygonal shaped and have a dense, phase bright center.

Differentiation

hES cells possess the ability to differentiate into many types of somatic cells. It is possible to make them differentiate into hematopoietic cells by co-culturing them with bone marrow stromal or endothelial cell lines (Kaufman et al., 2001). After differentiation, these cells express CD34, glycophorin A, CD15 and CD41, which are all expressed on hematopoietic cells. These cells could potentially be used therapeutically for transplantation and transfusion. hES cells will also differentiate into cardiomyocytes by co-culturing them with a visceral endoderm cell line, after which they acquire the appearance of beating muscle (Mummery et al., 2002). These differentiated cells could be used to restore cardiac function in damaged tissue. Also, hES cells can differentiate

into endocrine-like cells through directed developmental stages by using specific concentrations of growth factor-supplemented tissue culture medium for defined periods of time. In the final developmental stage of these endocrine-like cells they can secrete pancreatic hormones such as insulin, glucagon, somatostatin and pancreatic polypeptide (D'Amour et al., 2006). These cells could be used therapeutically in diabetes patients and other diseases associated with pancreatic dysfunction.

Importantly, ES cells can differentiate into neurons *in vitro*. This will be discussed in more detail below.

Induced Pluripotent Stem Cells

As mentioned previously, the number of hES cell lines available for distribution is limited. While many of these have been used extensively, the lines that have been most highly characterized are the first lines produced by Thomson et al. in 1998 including the H1, H7, H9, H13 and H14 lines. It would be ideal to have an unlimited number of lines to work with, but this has not been possible because of limitations on funding and the ethical concerns mentioned previously. In an interview with the New York Times on November 22, 2007, Dr. James Thomson said, "If human embryonic stem cell research does not make you at least a little bit uncomfortable, then you have not thought about it enough." For these reasons researchers have devised a method to transform somatic cells obtained from various sources into immortal, pluripotent stem cells.

History

The most commonly used method of de-differentiating somatic cells is to induce the expression of transcription factors Oct-4, SRY related HMG Box gene 2 (Sox-2),

kruppel-like factor 4 (Klf-4) and myelocytomatosis viral oncogene homolog (c-Myc) in the cell (Lowry et al., 2008, Maherali and Hochedlinger, 2008, Nakagawa et al., 2008, Park et al., 2007, Park et al., 2008a, Takahashi et al., 2007a). This was first accomplished in mouse cells in 2006 by Takahashi and Yamanaka using fibroblasts from embryos and adults, and the resulting cells were termed iPS cells. The expression of the aforementioned genes, among others, was induced in the cells using retroviral transduction, and utilizing tissue culture techniques and conditions similar to those used for hES cells. All four of these genes are involved in several transcriptional processes and feed-back loops, but some of the main functions related to iPS cell induction include: c-Myc and Klf-4 induce F-box protein 15 (Fbx-15) expression, which confers the ability of continual cell division; Oct-4 is required to maintain pluripotency; Sox-2 controls the expression of Oct-4. When a screening of 24 genes was performed, these four genes were found to be necessary and sufficient to convert fibroblasts into dividing pluripotent cells (Takahashi and Yamanaka, 2006).

The gene Nanog was originally hypothesized to be required for this process because of its role in maintaining pluripotency in early ES cells, but was subsequently found to be dispensable (Takahashi and Yamanaka, 2006). In addition, it was recently discovered that expression of the genes Oct-4 and Sox-2 in combination with Nanog and the RNA binding protein Lin-28 is also sufficient to reprogram cells (Takahashi et al., 2007a, Yu et al., 2007, Zhang et al., 2009). Also, it was found that expression of c-Myc makes cells more prone to errors during replication, and hence makes them more tumorigenic. However, the creation of iPS cells without c-Myc expression decreases the efficiency of this process dramatically (Nakagawa et al., 2008).

Viral Vectors

Attempts have been made to use adenoviral vectors and plasmid transfection to achieve stable expression of the genes required for reprogramming (Woltjen et al., 2009). However, it was found that stable and efficient endogenous expression of genes can be induced in most cell types utilizing a retroviral vector that contains the coding sequence of the desired gene. Retroviruses integrate into the genome of mitotic cells and use host-cell machinery to achieve transcription of their cellular components, as well as any genes inserted into their genome (Morita et al., 2000). However, in postmitotic cells a lentiviral vector is required to achieve integration. In this way the gene of interest is permanently integrated into the chromosomes of the target cells and provides unvarying expression of the gene *in vivo* (Tiscornia et al., 2006). Several different viral vectors have been used for this purpose including the murine leukemia virus (MuLV), respiratory syncytial virus (RSV) and cytomegalovirus (CMV) (Morita et al., 2000). Inducible retroviral and lentiviral vectors have also been used successfully for this purpose and are still used today, albeit less often than those utilizing a noninducible viral promotor (Maherali and Hochedlinger, 2008).

One problem with this method is that four different viral vectors must be integrated into the genome of a single cell in order to obtain expression of all four transcription factors that are necessary to make iPS cells. Since the efficiency of transduction is often low (0.001% to 0.01%), this is not always possible. Also, the potential for mutagenesis in the cells is increased with each integration event. Hence, a polycistronic lentiviral vector called pLentG-KOSM, which contains all four genes with 2A “self-cleaving” peptides driven by a CMV promoter, has been created is effective in

reprogramming mouse and human somatic cells (Carey et al., 2009, Shao et al., 2009). Using this construct it is possible to obtain iPS cells from a single integrated copy of the viral vector. In addition, a doxycycline-inducible polycistronic vector also containing all four transcription factors has been used to make iPS cells from human peripheral blood mononuclear cells (PBMCs) (Staerk et al., 2010). Again, since only one virus has to be successfully transduced into the cell, this should greatly increase the efficiency of reprogramming human and mouse cells.

In order to produce infectious virus, the viral constructs can be transfected into a packaging cell line, such as the 293T cell. Once a sufficient amount of virus is obtained it can be stored for long periods of time under the proper conditions and used for the subsequent infection of target cells.

Source Cells

Fibroblasts are relatively easy to obtain and culture, and grow well in ES cell culture conditions, which is why they are commonly used for reprogramming (Maherali and Hochedlinger, 2008). iPS cells can be generated from primary fibroblast lines that have been derived from skin punch biopsies from facial dermis or foreskin, or from commercially available, previously established lines (Park et al., 2008a). Also, it was shown that human synoviocytes obtained from synovial fluid can be used to generate iPS cells, although these cells are not commonly used for this purpose (Takahashi et al., 2007a). Human keratinocytes and adipocytes have also been used to create iPS cell lines (Carey et al., 2009, Sun et al., 2009). These cell types are more easily obtainable and tend to generate iPS cell lines more quickly than when fibroblasts are used. Importantly for this project, human B cells and monocytes have been used to create iPS cell lines

using doxycycline-inducible lentiviral vectors (Hanna et al., 2008, Hanna et al., 2009). Also, iPS cell lines have been created using CD34+ cells from mobilized peripheral blood as source cells (Loh et al., 2009). Recently investigators have found that T cells obtained from fresh or frozen peripheral blood can be used to generate iPS cells (Brown et al., 2010, Loh et al., 2010, Seki et al., 2010, Staerk, 2010). This has been accomplished using various lentiviral vectors. In most cases, the T cells have been expanded or stimulated *in vitro* before being infected with the relevant virus.

Feeder Cells

The generation and propagation of iPS cells requires a layer of feeder cells or other substrate, similar to the growth requirements for hES cells. Types of feeder cells that have been used in experiments where cells have been successfully reprogrammed include irradiated MEFs (Lowry et al., 2008), cells from mouse tail tips (Takahashi et al., 2007b) and SNL cells (an established fibroblast cell line) (Takahashi et al., 2007a). It is also possible to use the feeder-free system described above for ES cell culture utilizing Matrigel and mTeSR medium to generate iPS cells (Sun et al., 2009). This increases the potential for iPS cells to be used therapeutically because no contaminating mouse cells are used as a feeder layer, and no animal serum is used for culture.

Characterization

Characterization of iPS cells is similar to that of ES cells because reprogrammed cells should exhibit the same characteristics as ES cells. Hence, they should express the same markers, have similar morphology, have high telomerase activity, induce formation of teratomas and possess the same pluripotent capabilities. Indeed, successfully

reprogrammed somatic cells do express SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, alkaline phosphatase, Nanog, Oct-4, Sox-2, Klf-4, human telomerase reverse transcriptase (hTERT), reduced expression 1 protein (Rex-1) and growth differentiation factor 3 (GDF3) (Takahashi et al., 2007a, Lowry et al., 2008) among others as detected by RT-PCR. For the purpose of characterizing iPS cells, it is important to distinguish the endogenous expression of these genes from the retrovirus transgenes by the use of specifically designed primers. It has been proposed that live iPS cells should be stained for TRA-1-81 as a definitive marker for reprogrammed cells (Lowry et al., 2008). However, this method is technically difficult and RT-PCR and microarray analysis are still the preferred methods for marker expression.

iPS cells in culture form embryonic bodies (EBs) similar to hES cells in culture. These are compact, distinct spheres of cells with well defined borders that are segregated from the surrounding cells in the culture, especially fibroblasts that have not been transformed (Takahashi et al., 2007a, Lowry et al., 2008, Park et al., 2008a). The EBs are easily discernible from non-ES cell-like colonies by the trained observer because of the lack of a granular appearance (Takahashi et al., 2007a) and an appearance that seems to reflect light (Maherali and Hochedlinger, 2008). This distinctive morphology makes it possible to isolate the desired colony and re-plate the cells onto new culture dishes with feeder cells in order to expand them.

Similar to hES cells, iPS cells have high telomerase activity that can be detected using a commercially available assay that is based on the TRAP method (Savoysky et al., 1996). iPS cells have been shown to have doubling times similar to hES cells and are able to proliferate for up to 4 months (Takahashi et al., 2007a).

When implanted into SCID mice, iPS cells form teratomas that contain all three germ layers. The tissue types observed in the teratomas include retinal epithelium, neural rosettes, squamous epithelium, muscle, cartilage, bone, respiratory epithelium, gut epithelium, glandular tissue, adipose tissue and epidermis (Takahashi et al., 2007a, Park et al., 2008a). It is possible to confirm the presence of all three germ layers using RT-PCR to analyze the expression of glutamyl-tRNA amidotransferase chain A 4 (GATA4) which is expressed in endoderm, N-CAM which is expressed in ectoderm and Brachyury which is expressed in mesoderm (Park et al., 2007). The differentiation of iPS cells into cells of the germ layers is widely accepted as evidence that these cells possess developmental pluripotency.

Alternative Methods

Theoretically, methods other than those described above could be used to reprogram human somatic cells (Hochedlinger and Jaenisch, 2006). Nuclear transfer (a method used in cloning) has been attempted for this purpose in other species (Munsie et al., 2000, Yamanaka, 2007), but has yet to be tested in human cells due to ethical considerations. Somatic cell nuclear transfer consists of removing the nucleus from an oocyte, injecting the nucleus of a somatic cell, and then explanting the oocyte in culture where cells can proliferate. Using nuclear transfer, cell lines have been created that have ES cell characteristics including immortality, and the ability to form teratocarcinomas when transplanted into SCID mice, indicating pluripotency. Fusion of differentiated somatic cells with pluripotent ES cells has also been shown to give rise to hybrid cell lines that have characteristics of ES cells (Blau and Blakely 1999, Yamanaka 2007). However, in order for these cells to be used therapeutically, the ES cell nucleus would

need to be eliminated from the hybrid, and this has not yet been successfully accomplished. Also, incubation of somatic cells with cell extracts from oocytes and ES cells has been used as a cell free system of reprogramming in *Xenopus* cells (Kikyo et al., 2000). Another method of cell free reprogramming of somatic cells is to explant germline cells in culture. This has been accomplished using mouse testis cells with the addition of specific growth factors to the tissue culture media (Guan et al., 2006). These methods have all proven to be successful in other species for reprogramming somatic cells, but need to be tested in human cells. Unfortunately, embryos or oocytes are still required for nuclear transfer and fusion with ES cells. Currently, retroviral expression of the transcription factors discussed above is still the only successful method of inducing immortality and pluripotency in human somatic cells.

Differentiation

Most methods of driving the differentiation of iPS cells are similar to those used for ES cells. As with ES cells, usually iPS cells are cultured until they form EBs, and then dissociated and re-plated in differing culture conditions. Using this method and re-plating cells on methylcellulose with growth medium supplemented with hematopoietic cytokines, both myeloid and erythroid colony growth has been observed (Park et al., 2008a). By co-culturing iPS cells with a mouse stromal cell line (OP9 cells) derived from newborn calvaria (the superior part of the skull) differentiation into hematopoietic and endothelial cells has been observed. These differentiated cells have the phenotypes $CD34^{+}$ $CD43^{+}$ (hematopoietic precursors) and $CD31^{+}$ $CD43^{-}$ (endothelial cells) (Choi et al., 2009). Also, iPS cells have been successfully driven to differentiate into mature insulin-producing pancreatic cells by utilizing a chemically defined culture system

(Zhang et al., 2009a). Using this system, flow cytometry was used to show that 25% of the differentiated cells produce insulin in response to a glucose stimulus. In addition, adipocytes have been obtained from iPS cells by culturing EBs for 10 days on ploy-l-ornithine and fibronectin coated tissue culture plates with adipogenic medium consisting of 0.5 mM isobutylmethylxanthine (IBMX), 0.25 μM dexamethasone, 1 μg/ml insulin, 0.2 mM indomethacin and 1 μM pioglitazone (Taura et al., 2009). Transcription of adipose selective genes and lipid accumulation were used to prove the presence of adipocytes. Interestingly, EBs from iPS cells have been successfully directed to differentiate into functional cardiomyocytes that retain the ability to proliferate (Zhang et al., 2009b). This was accomplished by re-plating the cells on 0.1% gelatin-coated plates and using EB20 differentiation medium, which is standard ES cell medium containing 20% fetal bovine serum (FBS), l-glutamine, 2-mercaptoethanol and non-essential amino acids in DMEM/F12 media. Analysis of cardiac genes using RT-PCR, immunocytochemistry for cardiomyocyte markers, and electrophysiology to measure action potentials are the methods that were used to confirm a mature cardiac cell phenotype.

iPS cells can also be differentiated into functional neurons. This is discussed below.

Patient Specific iPS Cells

One great advantage of being able to use iPS cells therapeutically as opposed to ES cells is that fibroblasts could be obtained from specific patients, and after reprogramming could be transplanted back into the patient, thereby avoiding any possibility of rejection by the immune system. This provides a way for all patients to

receive functional cells or tissues to replace those ravaged by disease instead of just those who can be matched to a donor with a suitable human leukocyte antigen (HLA) haplotype. Indeed, successful reprogramming of patient-specific fibroblasts has been achieved (Dimos et al., 2008 and Park et al., 2008b). In 2008, Dimos et al. was able to successfully reprogram dermal fibroblasts from an 82-year-old female patient with amyotrophic lateral sclerosis (ALS). These cells were directed to differentiate into motor neurons which, theoretically, could be used for cell based therapy. The cells were not transplanted back into the individual in this case because of the persistence of the retroviruses and the presence of oncogenic genes used to reprogram the cells. Also, in Park et al. (2008b), either dermal fibroblasts or bone-marrow derived mesenchymal cells were taken from 10 patients with different diseases and were converted into stem cells. An iPS cell induction protocol similar to the one described above was used to establish cell lines that were specific for all 10 diseases including Duchenne muscular dystrophy, Becker muscular dystrophy, Down syndrome, Parkinson disease, juvenile diabetes mellitus and Huntington disease, among others. Much knowledge can be gained by allowing cells from these lines to differentiate into various tissues and studying the genetic abnormalities and pathologies that arise compared to normal tissue. Also, these abnormal tissues can be used for studying potential drug therapies.

Differentiation into Neurons

Human Embryonic Stem Cells

As mentioned previously, ES cells can differentiate into neurons *in vitro*, as was first described in mouse cells by Bain et al. in 1995. This was shown by the relatively

simple culture system of culturing ES cells for 4 days as aggregates and then adding retinoic acid to the medium and culturing for 4 more days. The resulting cells were found to express the mature neuron specific genes neurofilament (L subunit), brain-specific homeobox/POU domain protein 3 (Brn-3, a transcription factor), glutamate receptor subunits and glial fibrillary acidic protein (GFAP) as detected by real-time PCR (RT-PCR). Also, the differentiated cells express β-3 tubulin and neurofilament proteins and can generate action potentials, indicating functionality. This work led researchers to investigate the potential of hES cells to differentiate into neurons *in vitro*. Indeed, it was found that hES cells can differentiate into neural progenitor cells, which can further differentiate into the three neural lineages astrocytes, oligodendrocytes and neurons (Carpenter et al., 2001, Reubinoff et al., 2001, Schuldiner et al., 2001, Zhang et al., 2001).

Each investigator that published these results in 2001 used a slightly different method to drive differentiation of hES cells into neurons, but all began the process by allowing the hES cells to form EBs, dissociating the cells, and then re-plating the cells on tissue culture plates coated with various substrates. Carpenter et al. (2001), added mitogens and growth factors to the growth medium. These supplements include human epidermal growth factor (hEGF), human basic fibroblast growth factor (hbFGF), human platelet derived growth factor (hPDGF-AA), human insulin-like growth factor-1 (hIGF-1), human neurotrophin-3 (hNT-3) and human brain derived neurotrophic factor (hBDNF). In Reubinoff et al. (2001) a culture system consisting of media supplemented with hEGF and bFGF was used, while in Schuldiner et al. (2001) retinoic acid, β nerve growth factor (βNGF) and TGFβ1 was added to the culture media. In Zhang et al. (2001)

culture media was supplemented with insulin, transferrin, progesterone, putrescine, sodium selenite, heparin and fibroblast growth factor-2 (FGF-2). Therefore, different supplemental growth factors and combinations thereof will induce the differentiation of hES cells into neurons. In fact, one can obtain different types of neurons in this manner, including glutaminergic, *gamma*-aminobutyric acid (GABA)ergic, motor and sensory neurons. All of these different methods produce cells that express human neural progenitor and mature neuronal genes, including GFAP, β-3 tubulin, microtubule associated protein-2 (MAP-2), nestin and neural cell adhesion molecule (N-CAM) (Carpenter et al., 2001, Reubinoff et al., 2001). The differentiated cells express receptors for the neurotransmitters GABA, dopamine and serotonin. They can maintain a sodium and potassium current, and they can generate an action potential, indicating functionality (Carpenter et al., 2001, Schuldiner et al., 2001). Also, it was found that when the differentiated cells were transplanted into mice they migrated into various brain regions of the host (Zhang et al., 2001).

Culture conditions have recently been refined for conversion of hES cells into neurons; an efficiency rate of greater than 80% has been achieved through the inhibition of a family of signaling proteins, SMAD proteins, which are similar to both the Drosophila protein "mothers against decapentaplegic" (MAD) and the *C. elegans* protein SMA, using the small molecules Noggin and SB4431452 (Chambers et al., 2009). Therefore, the transforming growth factor beta (TGFβ) pathway seems to be important in this process. This represents an important advancement in the field that makes the culture of hES cells and differentiation into neurons more feasible and cost effective.

Dhara et al. published a paper in 2008 that outlines a specific method for creating

neural progenitor cells from hES cells using feeder-free cultures. hES cells are grown on Matrigel in three different defined medium preparations for 7 days at a time for 21 days without passaging the cells. Once neural progenitors are generated in this manner, cells may be further differentiated by continuing to culture for 14 to 21 days in medium lacking the pluripotency factors bFGF and LIF.

A reagent that is commonly employed in the culture and derivation of neurons is called neurobasal medium. This is a specific tissue culture media formulation that provides optimal growth conditions for rat embryonic neurons, as well as many other types of neurons in multiple species. Often neurobasal media is supplemented with a reagent called B27 instead of serum to promote the growth of hippocampal and other CNS neurons. There are 5 antioxidants contained in B27 supplement, as well as hormones, nutrients and other proteins. See Table 1 for a list of the components of B27 supplement. Also, a supplement called N2 is often used to promote the growth of neural stem cells, postmitotic neurons and tumor cells of neuronal phenotype. The components of N2 are listed in Table 1.

As an alternative to the growth factor supplemented media method, it is possible to drive differentiation of hES cells into neurons by co-culturing them with established cell lines (Schulz et al., 2003, Buytaert-Hoefen et al., 2004, Perrier et al., 2004). The cell lines that have been used for this purpose are the human hepatocarcinoma cell line HepG2 (Schulz et al., 2003) and the stromal cell lines MS5, S2 (Perrier et al., 2004) and PA6 (Buytaert-Hoefen et al., 2004). Also, co-culture with astrocytes has been shown to effectively drive differentiation (Buytaert-Hoefen et al., 2004).

It is also desirable to direct neuronal differentiation into specific subtypes for investigation in the laboratory and potential therapeutic use. Li et al. (2008) created motor neurons and ventral spinal progenitors from hES cells, and Perrier et al. in 2004 created midbrain dopamine neurons, indicating that it is possible to create specific subtypes of neurons *in vitro*.

Induced Pluripotent Stem Cells

Importantly, iPS cells can be directed to differentiate into neurons, and even specific neural subtypes by using the EB formation method discussed above for differentiation into other cell types (Takahashi et al., 2007b, Yu et al., 2007, Chamberlain et al., 2008, Dimos et al., 2008, Nakagawa et al., 2008, Park et al., 2008b, Chambers et al., 2009). This is achieved by supplementing the culture media with growth factors, similar to the protocols described above for hES cells (Dimos et al., 2008, Chambers et al., 2009). Supplementation with retinoic acid and a sonic hedgehog (SHH) agonist allows for the differentiation of iPS cells into motor neurons (Dimos et al., 2008). It is also possible to co-culture iPS cells with established cell lines to drive differentiation into neurons. This is done in a similar manner to that discussed above for hES cells.

Neural progenitor cells, mature neural cells and specific neuronal subtypes created from iPS cells have vast potential for therapeutic use in diseases such as Alzheimer's, Parkinson's, multiple sclerosis, spinal cord injury and amyotrophic lateral sclerosis (ALS), as well as traumatic injury to the central nervous system (CNS). Perhaps more importantly, these cells can be utilized in the laboratory setting to study differences in genetics, phenotype, development and treatment between cells obtained from diseased and healthy individuals.

Table 1. Components of B27 and N2 growth supplements

B27 Specifically for hippocampal and other CNS neurons	**N2** Specifically for neural stem cells, post-mitotic neurons and tumor cells of neuronal phenotype
d-Biotin, BSA, L-Carnitine HC, Corticosterone, Ethanolamine HCl, D-Galactose (Anhyd.), Insulin (Human, Zn), Linoleic Acid, Linolenic Acid, Progesterone, Putrescine.2HCl, Sodium Selenite (1000X), T-3/Albumin Complex, Transferrin (Human, Iron-Poor), Vitamin A Acetate, Vitamin E, Vitamin E Acetate, Superoxide Dismutase, Catalase, and Glutathione	Human Transferrin (Holo), Recombinant Insulin (full chain), Progesterone, Putrescine, Selenite

RESULTS AND DISCUSSION

Embryonic Stem Cells

iPS cells are equivalent to ES cells in many aspects, but ES cell culture techniques are well-defined as opposed to iPS cell culture techniques, and ES cell lines are commercially available. To establish a standard protocol for stem cell culture techniques in our laboratory and characterize stem cells through gene expression and extra-cellular protein markers, I have acquired the H9 (WA09) hES cell line from the National Stem Cell Bank. I have optimized the maintenance, expansion and preservation of these cells using the traditional feeder cell method (irradiated and mitomycin treated MEFs), as well as the serum-free feeder-independent system established by the Thomson lab in 2006 (Ludwig et al., 2006). Representative pictures of undifferentiated hES cells grown on Matrigel in mTeSR media are shown in Figure 1. Cells were grown according to protocols in the mTeSR user manual. I have expanded the cells in an undifferentiated state and cryopreserved them in liquid nitrogen for future use.

SSEA-1 is not expressed on the surface of undifferentiated hES cells and is up-regulated upon differentiation. However, in mouse cells SSEA-1 is down-regulated upon differentiation (Draper et al., 2002). SSEA-4 expression on the surface of hES cells is down-regulated as differentiation proceeds, whereas it is up-regulated on mouse cells upon differentiation (Draper et al., 2002). Oct-4 (also known as Pou5f1) is a widely used marker for undifferentiated hES cells because it is a transcription factor that is essential

for formation of the inner cell mass during development of the embryo (Nichols et al., 1998). It is also down-regulated upon differentiation of hES cells. TRA-1-60 and TRA-1-81 are also antigens that are expressed on the surface of undifferentiated hES cells that are down-regulated upon loss of pluripotency (Draper et al., 2002).

I confirmed the presence of these stem cell markers on hES cells, thus ensuring pluripotency, by analyzing proteins using flow cytometry. As expected, a relatively high percentage of H9 cells express the markers SSEA-4, Oct-4, TRA-1-60 and TRA-1-81, while the undifferentiated cells do not express the marker SSEA-1 (Table 2).

These results optimized ES cell culture techniques and characterized extracellular surface markers indicative of iPS cells in humans. This approach can be used on iPS cells, since iPS cells by definition express the same markers as ES cells.

Differentiation into Neurons

Dhara et al. (2008) show the derivation of neural progenitor cells from hES cells using feeder-free conditions. Briefly, I used "protocol 1" from Dhara et al. 2008 to create neural progenitor cells from H9 hES cells. This is done by harvesting hES cells using the normal mTeSR passaging protocol, and re-plating them at 2 x 10^5 cells per ml on 100 mm laminin coated plates in mTeSR media. Twenty four hours later, the medium is changed to Medium 1, (Table 3) and cells are incubated in this medium for 7 days without passaging, while changing the medium daily. The medium is then changed to Medium 2 and cells are grown for 7 days, after which the medium is changed to proliferation medium and cells are grown for 7 days. The cells can be passaged on day 22 for two to five passages. At this point, hES cells have been differentiated into human neural progenitors (hNP). The cells have an appearance that is similar to ES cells at this

stage of differentiation (Figure 2). Also, the hNPs have lost the high level of expression of the stem cell markers Oct-4, TRA-1-60, and TRA-1-81 that is observed in undifferentiated hES cells (Table 2). Also note that expression of SSEA-1 has increased, which indicates loss of pluripotency (Table 2).

Once hNPs are obtained, it is possible to further differentiate these cells into neurons by growing on laminin coated plates in proliferation medium without bFGF and LIF for an additional 14 days. bFGF and LIF are the factors that help retain pluripotency, and by removing them from the culture medium differentiation is accelerated. The cells acquire the appearance of neurons at the end of this additional 14 days of culture (Figure 3). Also, the differentiated cells have lost almost all expression of stem cell markers at this point, and SSEA-1 expression is further increased (Table 2).

Neuron marker gene expression was measured in all three cell types (hES, hNP and differentiated neurons) to show differentiation, by real-time PCR (Table 4). The gene nestin is an intermediate filament gene that is widely used as a marker for central nervous system progenitor cells (Dahlstrand et al., 1995). Nestin expression increased in hNPs and differentiated cells compared to ES cells. MASH1/achaete-scute homolog 1 (Mash1) is a transcription factor that promotes neuronal differentiation (Alvarez-Rodriguez & Pons, 2009), and neurogenic differentiation factor 1 (NeuroD1) is a transcriptional activator that is widely expressed in the development of the mammalian brain (Shida, et al., 2008). The expression of both of MASH1 and NeuroD1 increased dramatically from ES cells to differentiated neurons. Growth associated protein 43 (GAP43) is a membrane-bound phosphoprotein highly expressed by developing neurons, and also expressed by regenerating neurons (Nguyen et al., 2009). Expression of GAP43

also dramatically increased in differentiated neurons compared to ES cells. Microtubule associated protein 2 (MAP2) is the major microtubule protein found in brain tissue, and B-3 tubulin is the major building block of the microtubules found in the brain (Carpenter et al., 2001, Schuldiner et al., 2001). MAP2 was not analyzed using the real-time PCR, but protein expression was analyzed and will be discussed below. Interestingly, B-3 tubulin gene expression did not change dramatically, but protein expression is markedly higher in hNPs and differentiated neurons compared to ES cells (Table 5). Neuronal nuclei (NeuN) is a DNA-binding nuclear protein found in most neuronal cell types in vertebrate neurons (Kempermann et al., 2004). NeuN has only recently been identified as hexaribonucleotide-binding protein 3 (Fox3), which belongs to the Fox-1 family of splicing factors (Kim et al., 2009). NeuN, gene expression decreased from the hES cell stage to the differentiated neuron state, although protein expression increased (Table 5). Gene expression does not always correlate with protein expression and function.

To further examine gene expression, microarray analysis was performed using Agilent's 44,000 human gene expression array with RNA extracted from three biological replicates each of hES cells, hNPs, differentiated neurons, and mature human fetal neurons acquired from a commercial source used as a control. Controls included in the mircroarray indicate that the labeled material was of high quality, and the hybridization technique was superior, such that no normalization was required for the data set. A heat map shows that the distribution of over- and under-expressed genes in each sample was very similar for each biological replicate in all sample sets (Figure 4). This heat map also shows that each cell type arising from the hES cells (ie. hNPs and differentiated neurons)

has an expression pattern that is more similar to hES cells than the mature neurons used as a control.

The microarray data set was analyzed using GeneSifter software. When a T-test was performed with a p value cutoff of 0.001 using Benjamini and Hochberg analysis, it was found that there are 3,942 genes that have at least a five fold difference in expression (including both over- and under-expressed genes) between the control neurons and hES cells. When using the same t-test conditions, it was found that there are 3,078 genes that have at least a five fold difference in expression between control neurons and hNPs, and 1,981 genes that have at least a five fold difference in expression between control neurons and differentiated neurons. Thus, as the cells differentiate into neurons *in vitro* using the protocol outlined above, the pattern of gene expression becomes similar to human fetal neurons and less like hES cells.

Next, individual genes from the microarray data set were examined to determine the changes in expression using control neurons as a reference. For the ES cell markers there is a large difference in gene expression between control neurons and ES cells, a smaller difference in gene expression between control neurons and hNPs, and the smallest difference in gene expression is observed between control neurons and differentiated neurons (Figure 5). Unfortunately, it is not known what genes encode the proteins that are detected by the antibodies directed against SSEA-4 and TRA-1-60, so it was not possible to perform analyses for these genes. The analysis described above for ES cell markers was also performed on the microarray data set for neuron markers (Figure 6). For the majority of neuron markers examined, the difference in gene expression compared to control neurons decreased as cells differentiated from hES cells

to differentiated neurons. This suggests that cells become more like mature neurons in the proteins they express as they develop from ES cell to differentiated neurons.

The standard approach to confirm iPS cells is by fixing cells and subsequently performing immunohistochemistry with antibodies that recognize the gene of interest (Loh et al., 2010, Staerk et al., 2010, Warren et al., 2010). A limitation of this method is that it only provides the researcher with a positive or negative (qualitative) result. However, when protein expression is measured by flow cytometry, the researcher obtains qualitative as well as quantitative data because information is obtained indicating the number of cells that are positive for a given marker and the level of expression of the marker. For this reason, this study measures the expression of neuronal marker proteins using flow cytometry with antibodies directed against the genes of interest (Table 5). Importantly, the levels of Beta-3 tubulin and NeuN increase from ES cells to hNPs to differentiated neurons, and the other neuronal markers are present in sufficient quantities to ensure function.

Because transcription of ES cell markers decreases so markedly, and neuronal marker gene expression and protein presence increase after differentiating ES cells, these results clearly demonstrate that the majority of ES cells have successfully differentiated into neurons. Using the protocol outlined above, a method has been established to culture and create iPS cells from human PBMCs.

Induced Pluripotent Stem Cells

As stated above, four transcription factors are critical for de-differentiation of somatic cells into iPS cells. Therefore, I utilized the aforementioned commercially available lentiviral vector called pLentG-KOSM, which supposedly expresses all four

transcription factors necessary to create iPS cells, to make iPS cells using human fibroblasts and MEFs. There are many advantages to using this vector in that the pLentG-KOSM construct contains all four relevant transcription factors in a single open reading frame, transcription is controlled by a CMV promoter, the peptides are self-cleaving 2A peptides, and an internal ribosomal entry site (IRES) driven GFP reporter is present (Figure 7). This vector has been used by two laboratories to create iPS cells from both human and mouse source cells (Carey et al., 2009 and Shao et al., 2009).

Producing infectious KOSM lentivirus was done by co-transfecting 293FT cells (human embryonal kidney cells transformed with the SV40 large T antigen) with the KOSM plasmid, along with the pCMVΔR8.2 packaging plasmid (Figure 8) that encodes Gag and Pol proteins, and the pCMV-VSV-G envelope plasmid (Figure 9) that encodes VSV-G pseudotyped protein. This was accomplished using a calcium phosphate transfection method. Supernatant which contains viral particles, was collected 48 hours post-transfection and subsequently concentrated by ultracentrifugation at 50,000 RPM for two hours at four degrees Celsius (C). Viral preparations were stored at -80 degrees C until use without loss of infectivity.

Virus infectivity was determined by incubating a 1:10 dilution of virus on HeLa cells (human cervical cancer cells) for 8 hours, and then harvesting cells 48 hours postinfection. The percentage of infected cells was determined using flow cytometry to detect GFP fluorescence (Figure 10). Using this method, it was found that almost 100 percent of cells were infected with the KOSM virus. The titer of the virus was determined to be 5.85×10^8 infectious units per milliliter (ml) by using the equation (F x

C_0 / V) x D, where F is percentage of cells infected, C_0 is total number of target cells at time of infection, V is volume of inoculum, and D is the dilution factor of the virus.

After confirming the presence of infectious KOSM lentivirus, validation of the efficacy of the system was performed by creating iPS cells in human fibroblasts. Human fetal lung fibroblast cells, called IMR90 cells, were infected with the KOSM virus. IMR90 cells have been used to make iPS cells previously using a different lentivirus system (Yu et al., 2007). Using a 1:10 dilution of KOSM, an infection rate close to 100 percent was obtained as determined by flow cytometry to detect GFP (Figure 11). Forty-eight hours postinfection the cells were trypsinized and plated on a monolayer of irradiated MEF feeder cells. Standard human ES cell tissue culture medium (DMEM/F12, 20 % Knockout Serum Replacer, 1 mM l-glutamine, 100 mM 2-mercaptoethanol, 2 mM nonessential amino acids, 0.25 μg/ml bFGF) was used to feed the cells, and was exchanged daily. Cells were observed for at least 30 days post-infection. No iPS colonies were observed at any time postinfection. In addition, MEFs were infected in the same manner as described above, plated on irradiated MEF feeder cells, and incubated with mouse ES cell tissue culture medium which was exchanged daily. After at least 30 days postinfection, no iPS colonies were observed.

Because cells infected with the KOSM lentivirus failed to make iPS colonies, plasmid DNA was analyzed to ensure that the plasmid was intact. Undigested KOSM plasmid DNA is approximately 13 kilobases (Kb). After digesting 1 microgram of KOSM plasmid DNA with the restriction endonucleases Not I and Rsr II, bands of the predicted sizes, 3 Kb and 10 Kb (Figure 12) were observed. This indicates that the plasmid is intact and should contain all necessary components to make iPS cells.

After determining that the plasmid DNA was intact, confirmation was done showing that cells infected with the KOSM lentivirus were producing the transcription factors that are encoded in the genome by doing western blots (Figure 13). Briefly, MEFs were infected with a 1:10 dilution of the virus and harvested three days post-infection, along with uninfected MEFs. Protein was extracted from cell lysate using protein extraction buffer (18 mM HEPES, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF and protease inhibitor cocktail) and quantified using the Bradford protein assay. Proteins were separated by polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes overnight. Membranes were blocked in 5% milk and then incubated with optimized dilutions of antibodies directed against Klf-4, Oct-4, Sox-2 and c-Myc. Protein from mouse ES cells was used as a positive control for the antibodies against the transcription factors. An antibody that detects mouse GAPDH was used as a loading control. All samples contained similar amounts of protein, and a low level of Klf-4 was observed in both infected and uninfected cells. However, no detectable level of Oct-4, Sox-2 or c-Myc was observed in either the infected or uninfected cells. This indicates that cells infected with the KOSM lentivirus are not producing the proteins encoded in the plasmid that are necessary to make somatic cells differentiate into iPS cells.

Because the KOSM lentivirus system was not able to produce iPS cells, an alternative method was used. In 2010, work was published from the Jaenisch lab indicating that iPS cells had been created from human PBMCs (Staerk et al., 2010). This was accomplished using a doxycycline-inducible polycistronic lentivirus called

pHAGE2-TetOminiCMV-hSTEMCCA (Figure 14) that encodes all four transcription factors. The proteins are self-cleaving 2A peptides, and an internal ribosome entry site (IRES) drives expression of the two peptides that are farthest downstream from the doxycycline-inducible promoter. This vector is used in conjunction with a lentivirus called FUW-M2rtTA that contains a reverse tetracycline transactivator (Figure 15) which is driven by the ubiquitin C promoter, making it constitutively active. Unfortunately we are unable to directly determine if cells are infected by either vector because there is no reporter present. However, it is possible to verify infection by performing PCR to ensure increased transcription of the four transcription factors, or performing flow cytometry or western blots with antibodies directed against the four transcription factors to detect increased levels of protein.

When cells are co-infected with both plasmids and doxycycline is present, transcription is initiated and the four peptides should be transcribed. PBMCs are infected using a method called "spin-infection" (Staerk et al., 2010). With this method, cells are centrifuged with dilutions of both viruses four times for 45 minutes at 1600 RPM. Infected cells are then placed on a layer of irradiated MEFs and incubated with ES cell tissue culture medium containing 2 μg/ml doxycycline. Using this method iPS colonies should appear 25-40 days after infection.

To test this system, both viruses were produced in 293FT cells using a calcium phosphate method of transfection and concentrated as described above. A monolayer of MEFs was infected using a 1:10 dilution of both viruses. The day following infection the tissue culture medium was changed to standard MEF medium containing 2 μg/ml doxycycline. Four days after infection iPS colonies were seen in abundance on infected

MEFs, while no iPS colonies were observed on uninfected MEFs incubated with the same tissue culture medium (Figures 16 and 17). In order to characterize the iPS cells and to further validate this lentivirus system, cells from plates with iPS cells were harvested and western blots were performed to ensure presence of the four transcription factors (Figure 18). Protein from infected and uninfected MEFs was analyzed as described above. A similar amount of total protein is present in each sample as determined by the level of GAPDH. The level of protein is increased in infected cells for all four transcription factors except Klf-4, where the level of protein is similar between infected and uninfected cells. This shows that cells infected with both viruses are producing the transcription factors necessary to make iPS cells.

This work presented herein has shown that it is possible to create iPS cells using the pHAGE2-TetOminiCMV-hSTEMCCA lentiviral vector in conjunction with the FUW-M2rtTA vector. Hence, PBMCs will be coinfected with both viruses and plated on a layer of feeder cells to see if iPS colonies form.

Table 2. Flow cytometry for ES cell markers on cells in various stages of differentiation into neurons. The number listed in the table is the percentage of cells that are positive for each antibody in the sample. All antibodies were purchased from StemCell Technologies. All antigens are expressed on the surface, except for Oct-4 which is internal. All primary antibodies were produced in a mouse, and SSEA-1, Oct-4 and SSEA-1 are IgG, while TRA-1-60 and TRA-1-81 are IgM. Fluorescein (FITC)-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-mouse IgM were used as secondary antibodies.

ES Cell Markers	**SSEA-4**	**Oct-4**	**SSEA-1**	**TRA-1-60**	**TRA-1-81**
ES cells	**77%**	**82%**	**0**	**20%**	**40%**
Neural Progenitors	**70%**	**2%**	**5%**	**1%**	**0**
Differentiated Neurons	**4%**	**7%**	**7%**	**1%**	**2%**

Table 3. Components of tissue culture medium used for differentiating ES cells into neurons. Each type of medium is filter sterilized before use. Medium is changed daily throughout the duration of the protocol.

Medium 1	Medium 2	Proliferation Medium
DMEM/F12 20% Knock-out Serum Replacement 1% Nonessential amino acids 1 mM l-glutamine 1x penicillin/streptomycin	DMEM/F12 1x N2 supplement 4 ng/ml hbFGF 2 mM l-glutamine 1x penicillin/streptomycin	Neural Basal Medium 1x B27 supplement 20 ng/ml hbFGF 10 ng/ml LIF 2 mM l-glutamine 1x penicillin/streptomycin

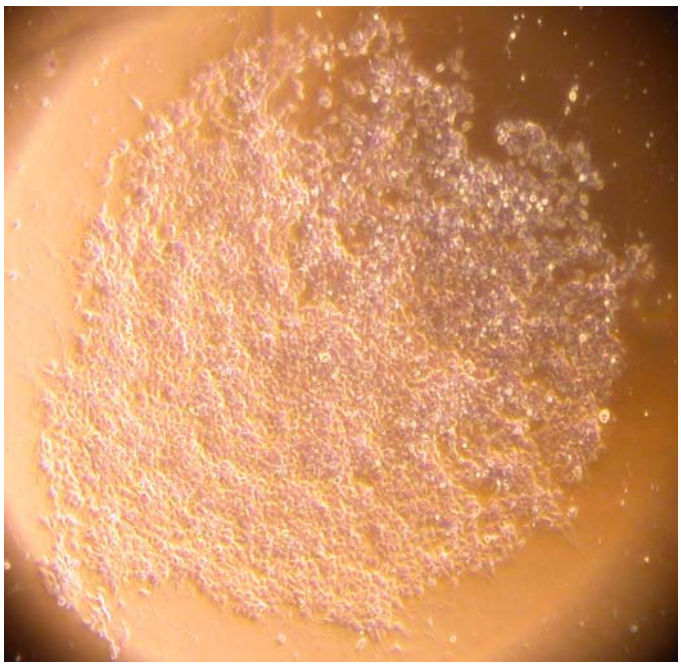
Table 4. Results from real-time PCR for neuron markers. PCRs were performed on a Light Cycler 480 (Roche) using SYBR Green chemistry. Samples were run in triplicate and normalized to β-actin. Primers were designed using Primer3 software (or other software programs available for public use) and synthesized by the DNA/peptide core facility at the University of Utah. RNA was extracted using a Qiagen RNeasy kit, and cDNA was synthesized using M-MLV reverse transcriptase.

Neuron Markers	**Nestin**	**Mash1**	**B III Tubulin**	**NeuroD1**	**GAP43**	**NeuN**
ES cells	**1.8**	**1.4**	7.4	**1.5**	**2.7**	12.6
Neural Progenitors	**3.7**	**5.1**	3.5	**31.1**	**9**	1.5
Differentiated Neurons	**4.9**	**24.2**	5.8	**15**	**30.8**	1.1

Table 5. Flow cytometry for neuron markers on cells in various stages of differentiation into neurons. SK-N-SH cells (a neuroblastoma cell line) were used as a positive control for the flow cytometry. The number listed in the table is the percentage of cells that are positive for each antibody in the sample. All antibodies were purchased from Abcam, except for NeuN, which was purchased from Invitrogen. All antigens are intracellular, so 0.1% Triton X-100 was used to permeabilize cells. Nestin, B-3 tubulin and NeuN primary antibodies were produced in a mouse, while MAP2, NeuroD1 and GAP43 primary antibodies were produced in a rabbit. FITC-conjugated goat anti-mouse and FITC-conjugated donkey anti-rabbit were used as secondary antibodies.

Neuron Markers	**Nestin**	**MAP2**	**B III Tubulin**	**NeuroD1**	**GAP43**	**NeuN**
SK Cells	90%	92%	84%	99%	99%	91%
ES cells	**3%**	**84%**	**16%**	**83%**	**13%**	**33%**
Neural Progenitors	**5%**	**61%**	**36%**	**81%**	**18%**	**32%**
Differentiated Neurons	**5%**	**28%**	**80%**	**71%**	**7%**	**49%**

A



B

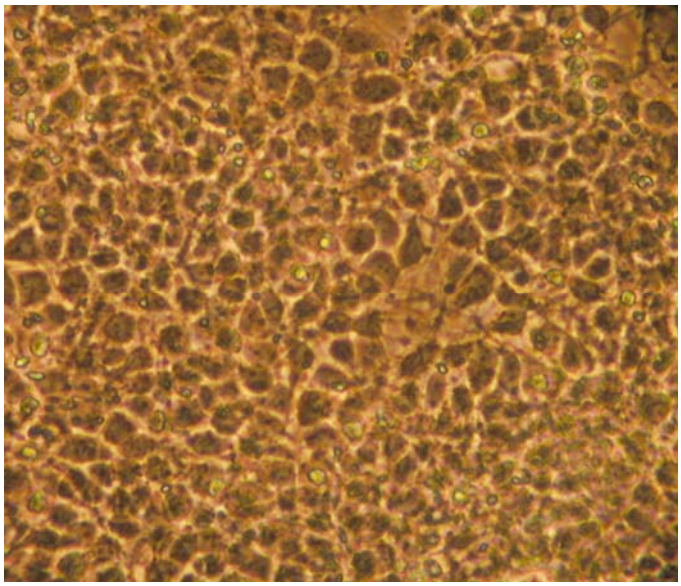
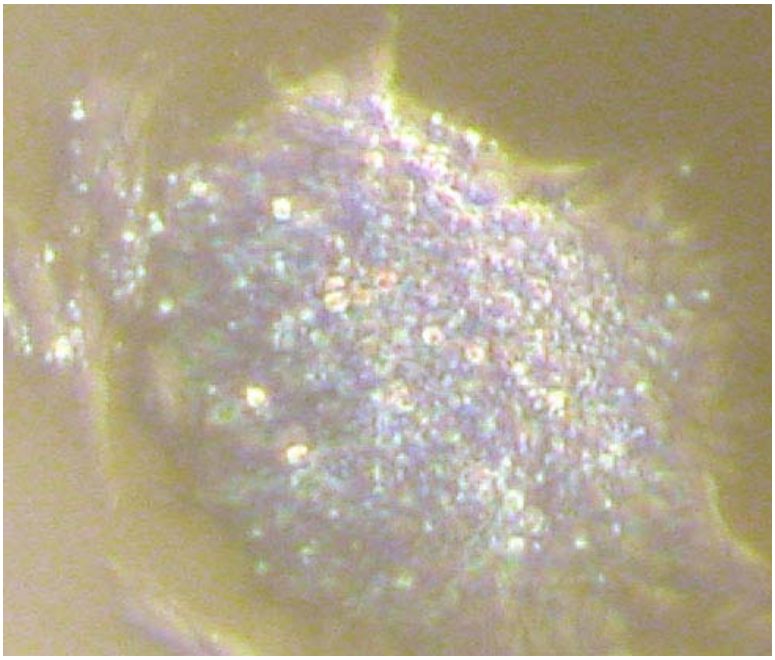


Figure 1. Undifferentiated hES cells grown on Matrigel in mTeSR media. A, 4x magnification. B, 20x magnification.

A



B

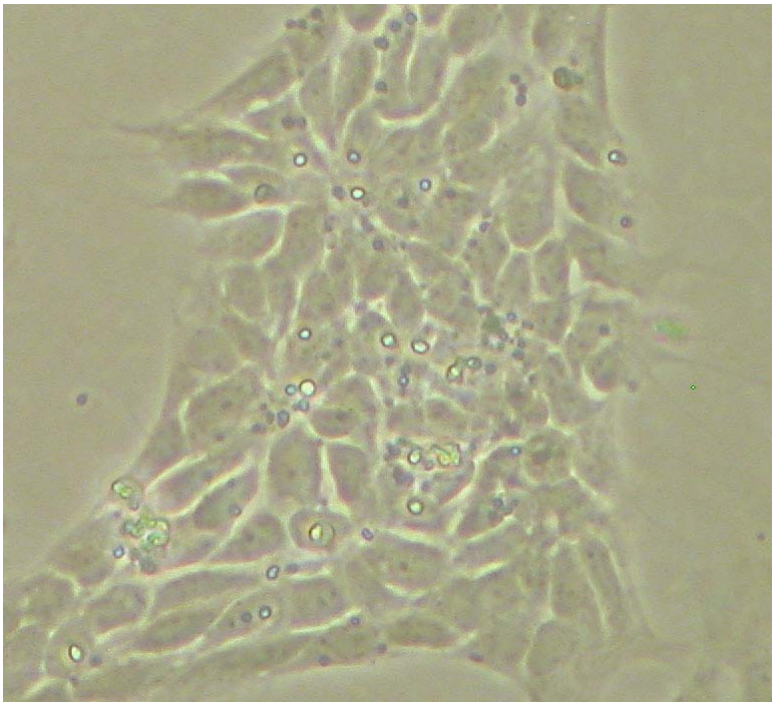


Figure 2. Human neural progenitors grown on laminin coated plates in proliferation media. A, 4x magnification. B, 20x magnification.

A

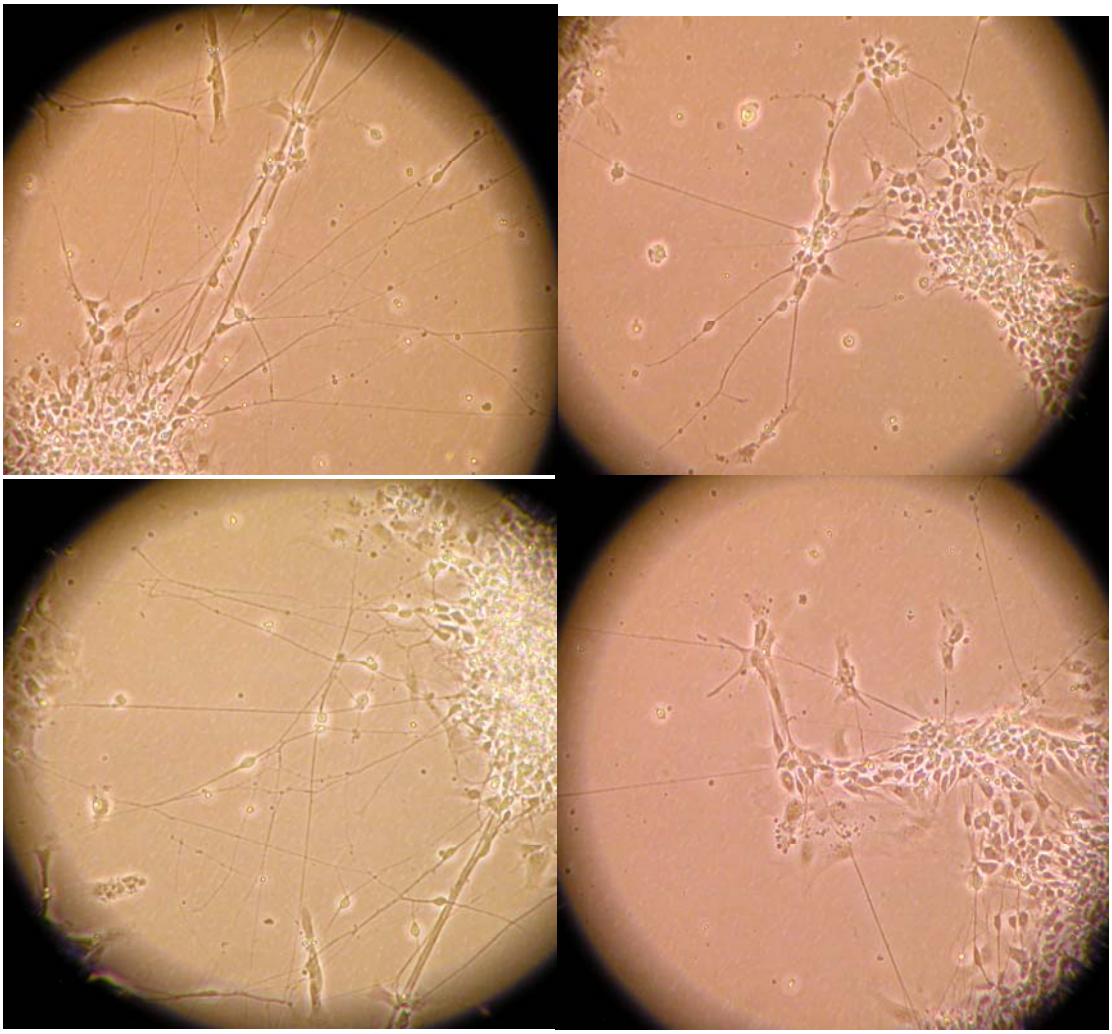


Figure 3. Human ES cells that have been differentiated into neurons, grown on laminin coated plates in proliferation media lacking bFGF and LIF. A, 20x magnification. B, 40x magnification. C, for comparison, human fetal neurons cultured for 6 days, 20x magnification.

B

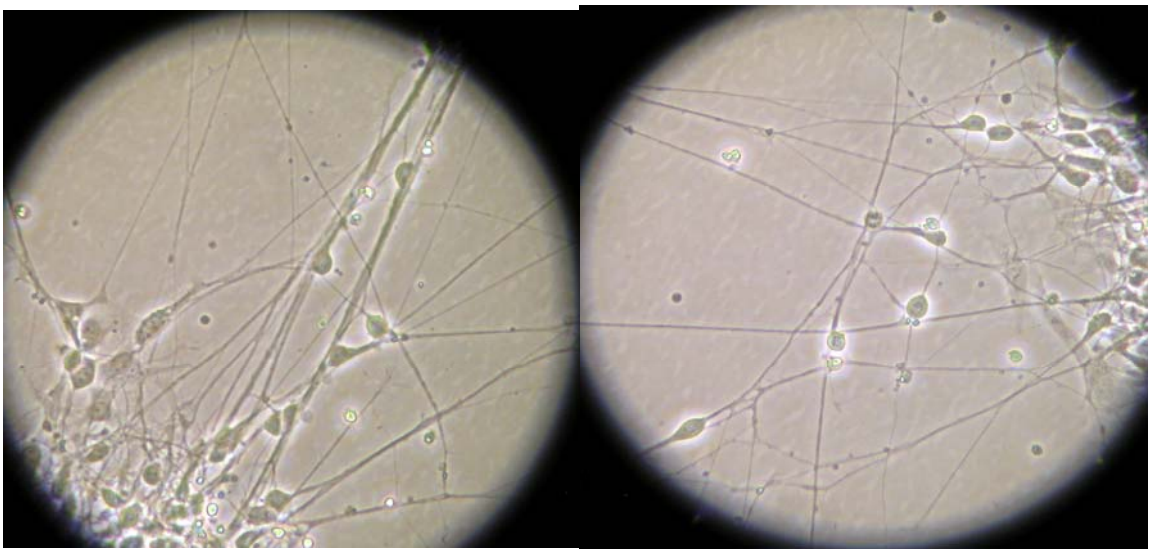


Figure 3, continued.

C

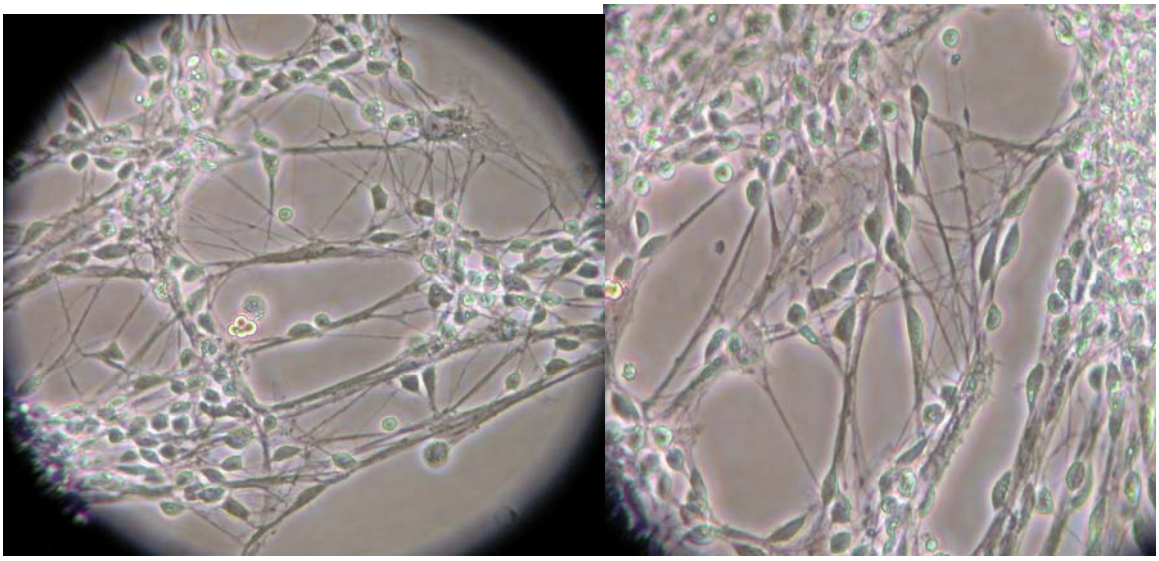


Figure 3, continued.

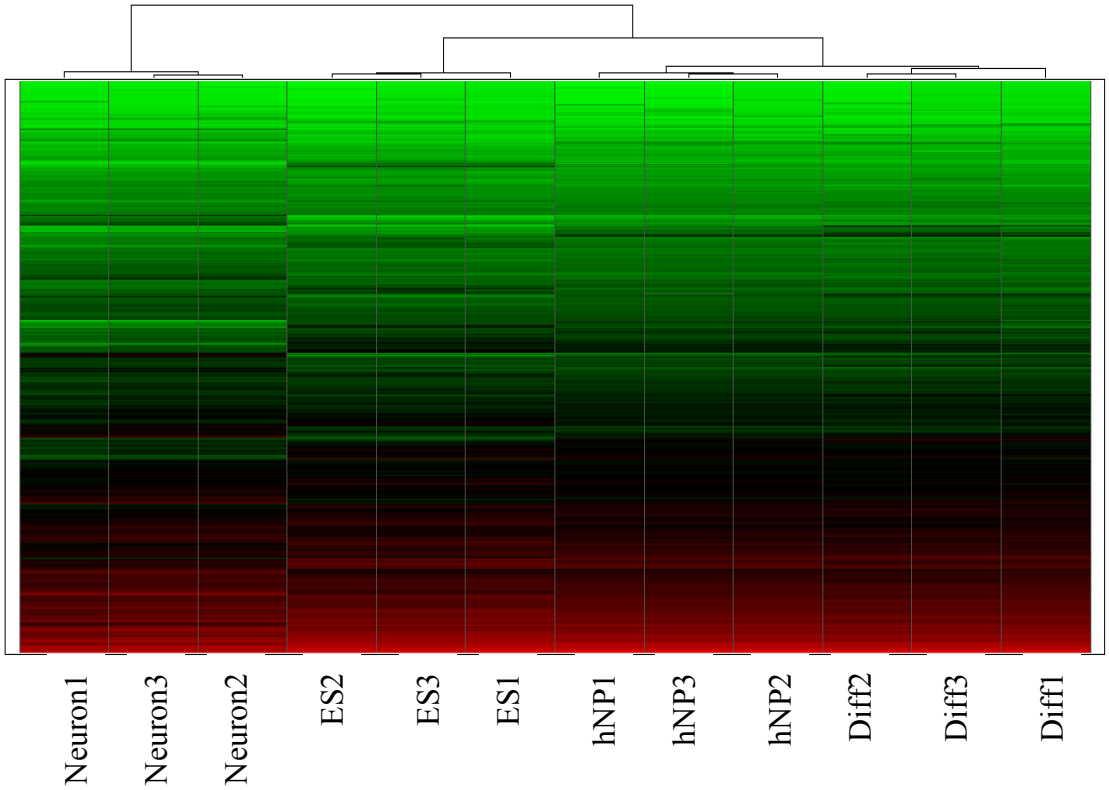


Figure 4. Heat map of over- and under-expressed genes from microarray. These results were generated from the Agilent 44,000 human gene expression array performed as described in the text. Green represents genes expressed at low levels, and red represents genes expressed at higher levels.

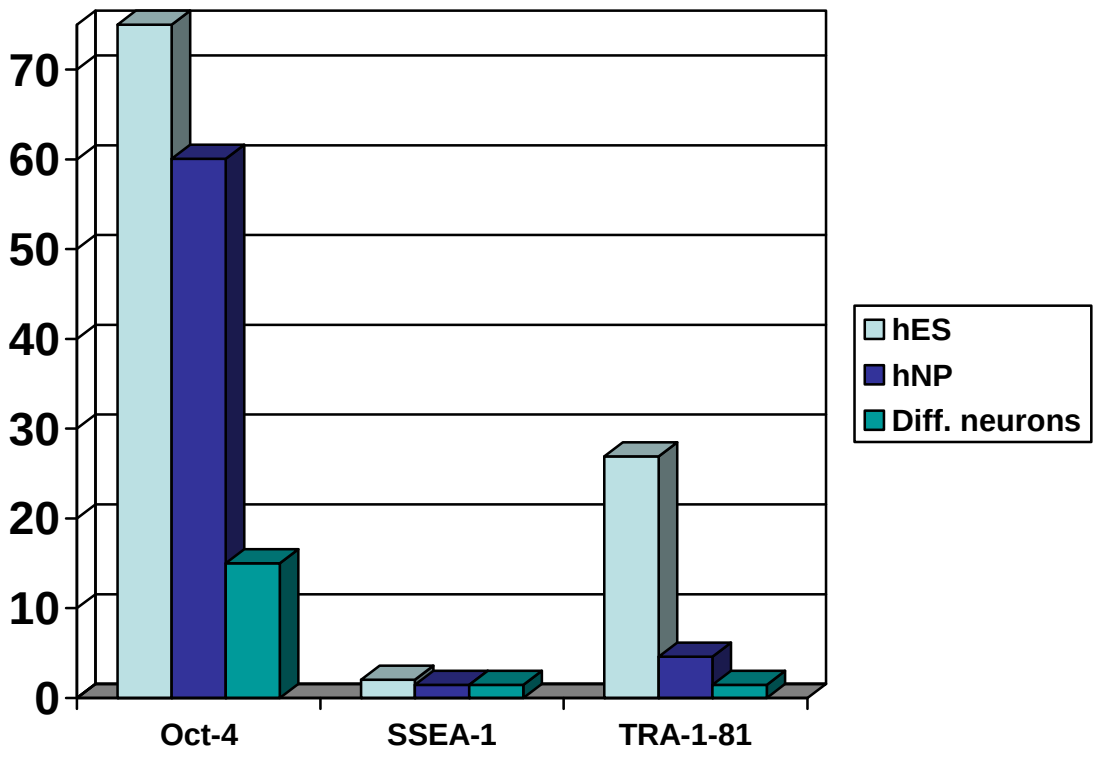


Figure 5. Fold change in ES cell marker gene expression determined from the microarray data set. Data were analyzed using GeneSifter software with control neurons as the reference value. Diff. neurons: differentiated neurons. Note: Fold difference between control neurons and hES cells for Oct-4 is 1843.

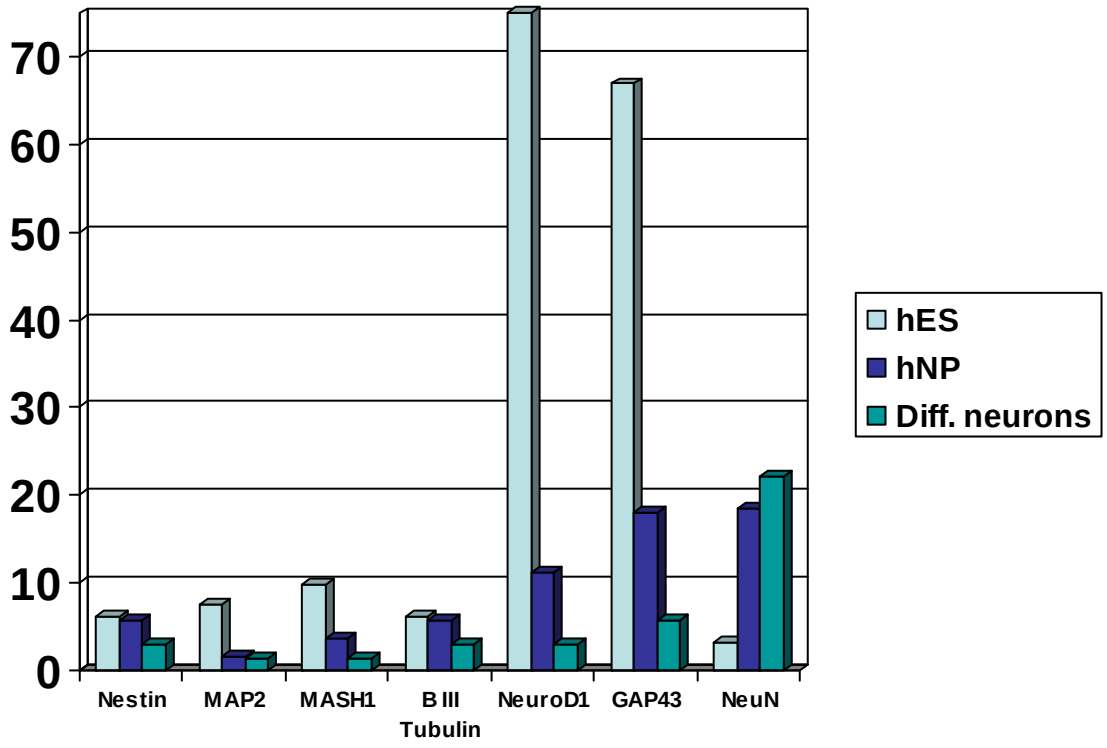


Figure 6. Fold change in neuron marker gene expression determined from the microarray data set. Data were analyzed using GeneSifter software with control neurons as the reference value. Diff. neurons: differentiated neurons. Note: Fold difference between control neurons and hES cells for NeuroD1 is 161.

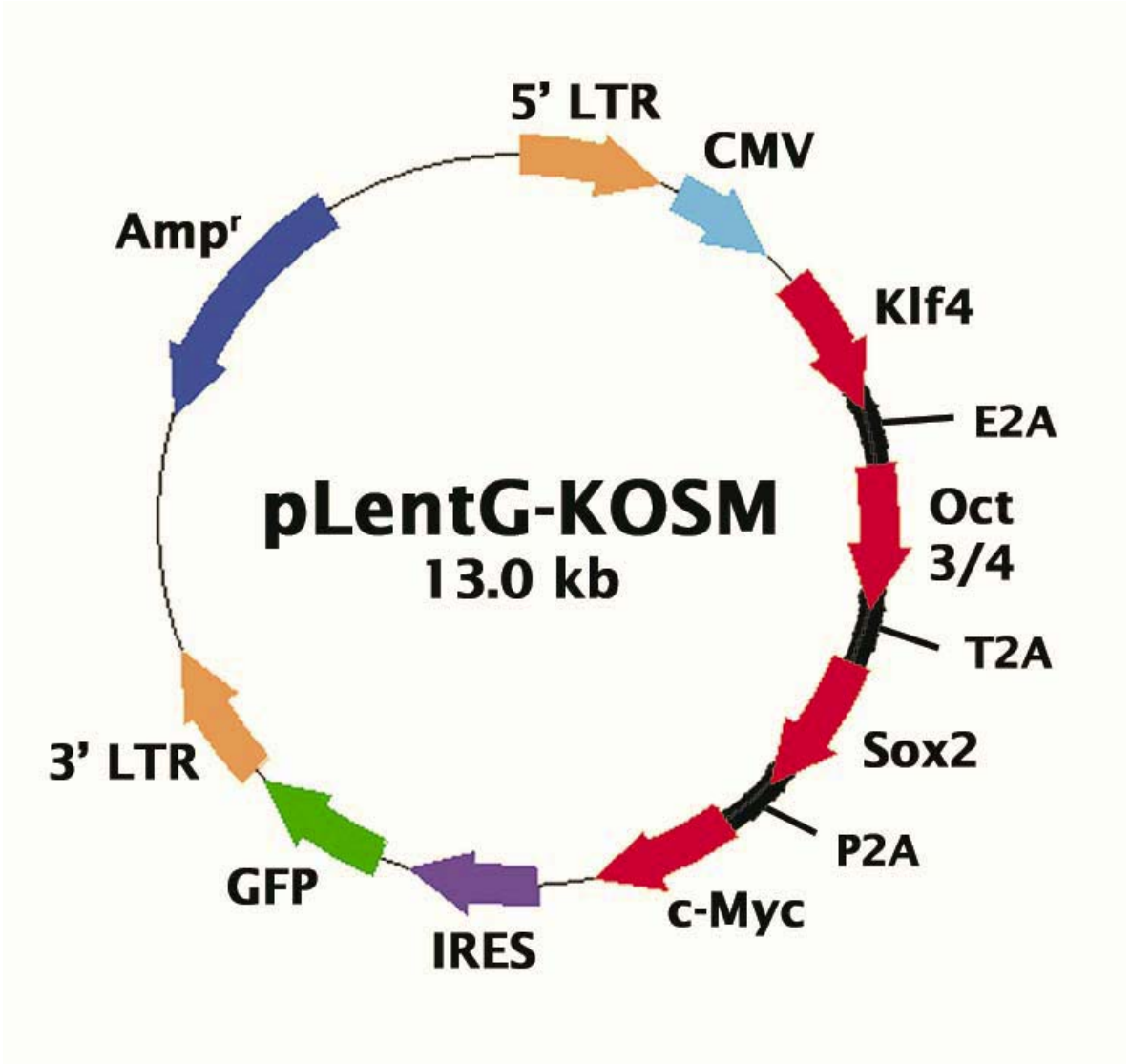


Figure 7. Schematic representation of the pLentG-KOSM vector. Image was obtained from Cell Biolabs, Inc.

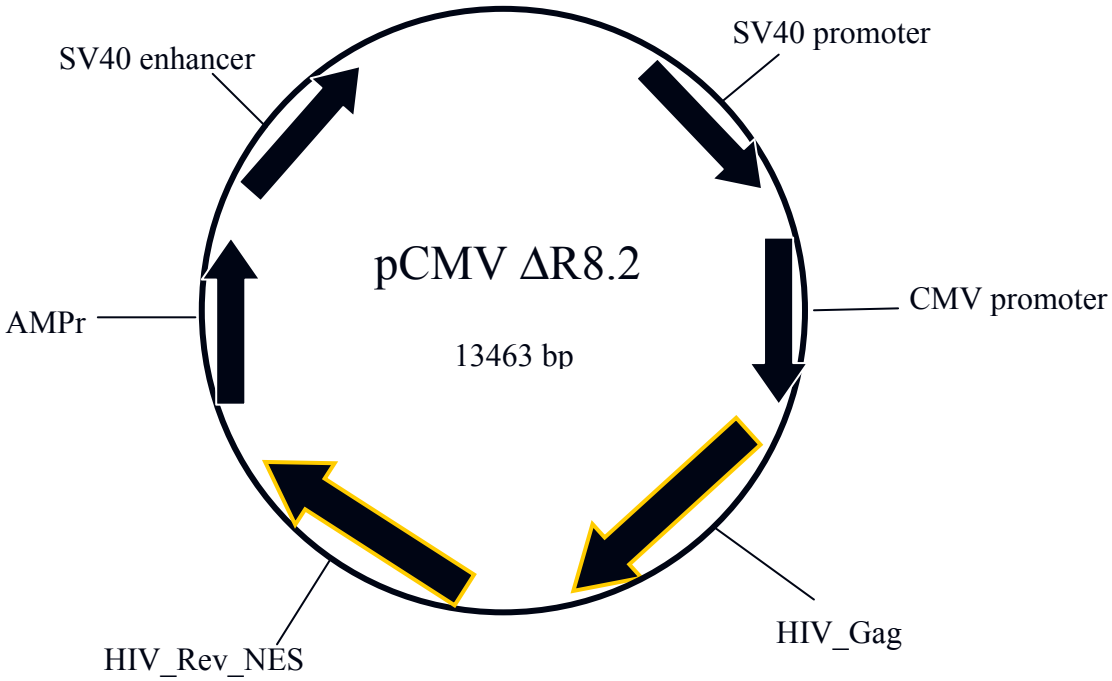


Figure 8. Schematic representation of the pCMVΔ8.2 vector.

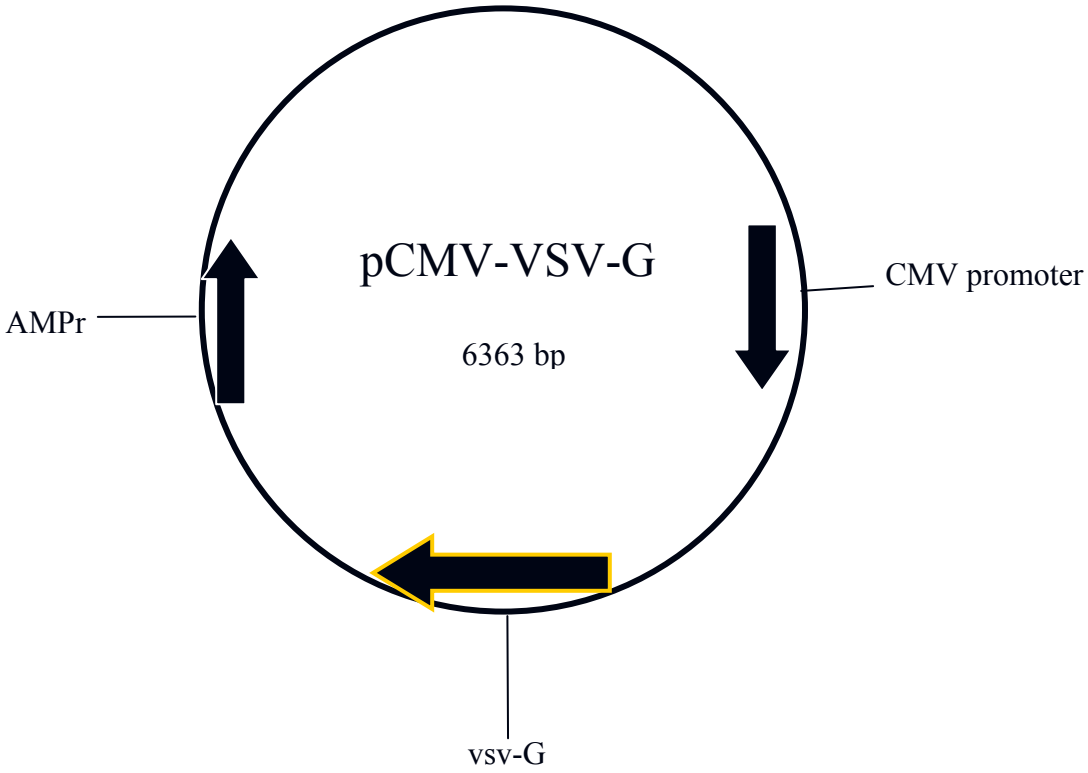


Figure 9. Schematic representation of the pCMV-VSV-G vector.

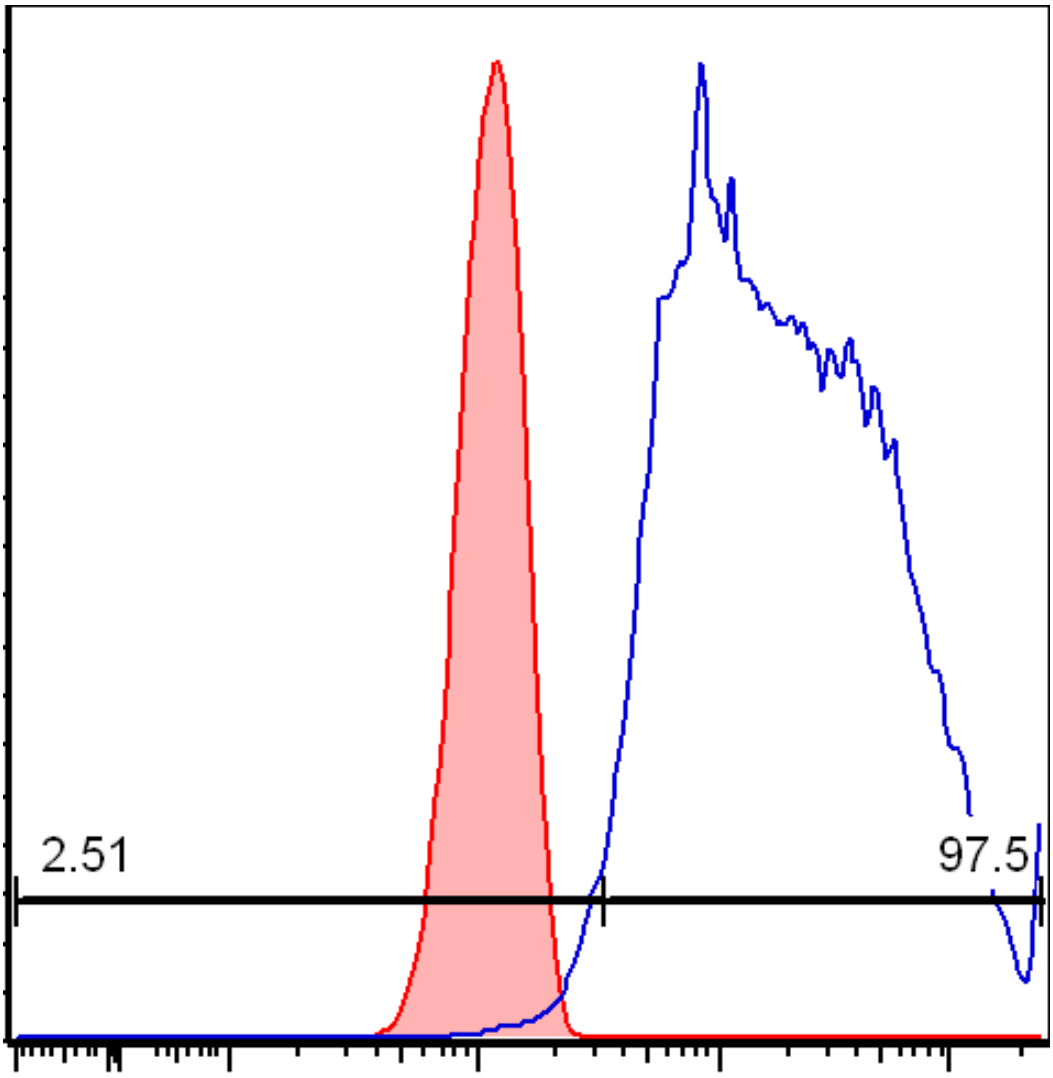


Figure 10. Flow cytometry on HeLa cells 48 hours postinfection. X-axis represents increasing GFP fluorescence. Pink histogram represents uninfected cells, and blue histogram represents infected cells.

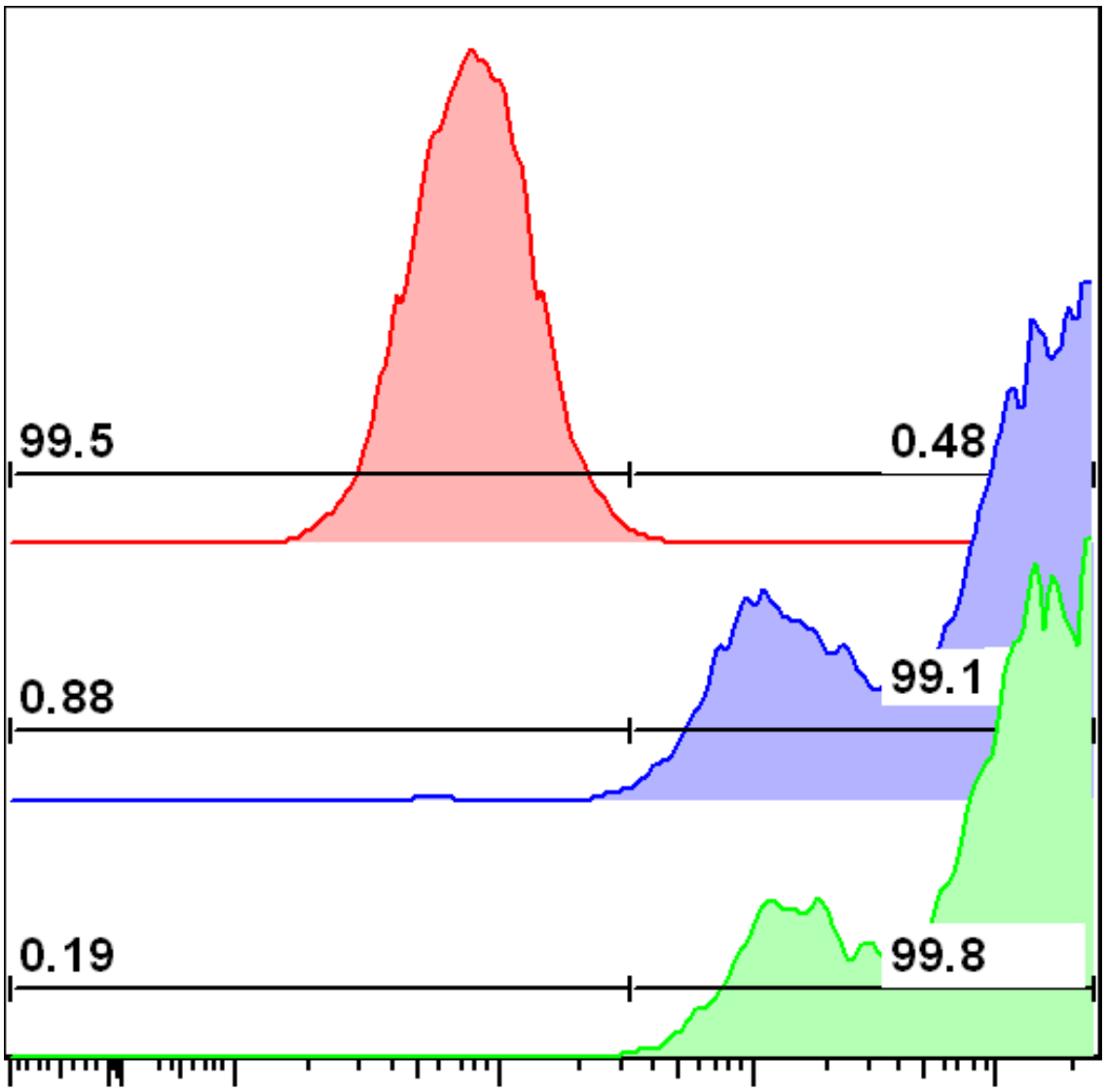


Figure 11. Flow cytometry on IMR90 cells 48 hours postinfection. X-axis represents increasing GFP fluorescence. Pink histogram represents uninfected cells and blue and green histograms represent duplicate wells of infected cells.

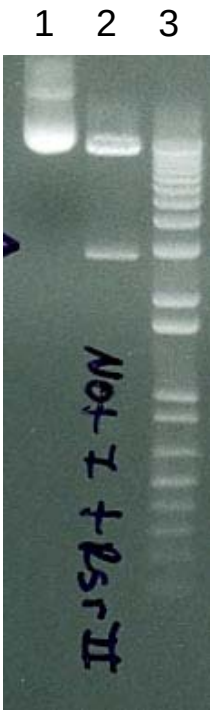


Figure 12. Agarose gel showing KOSM plasmid DNA digested with Not I and Rsr II. Lane 1, uncut KOSM plasmid DNA. Lane 2, 1 μg of KOSM plasmid DNA digested with Not I and Rsr II. Lane 3, nucleic acid marker.

Figure 13. Western blot using protein from mouse ES cells, uninfected MEFs and infected MEFs.

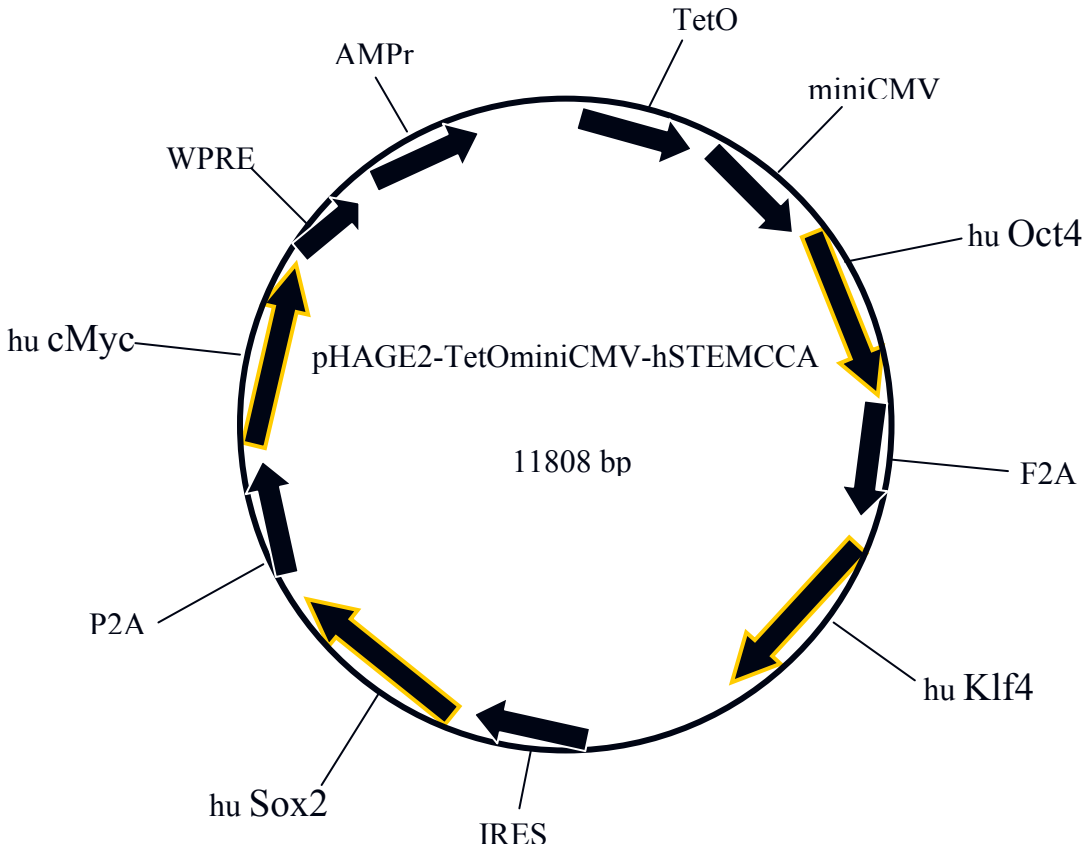


Figure 14. Schematic representation of the pHAGE2-TetOminiCMV-hSTEMCCA vector.

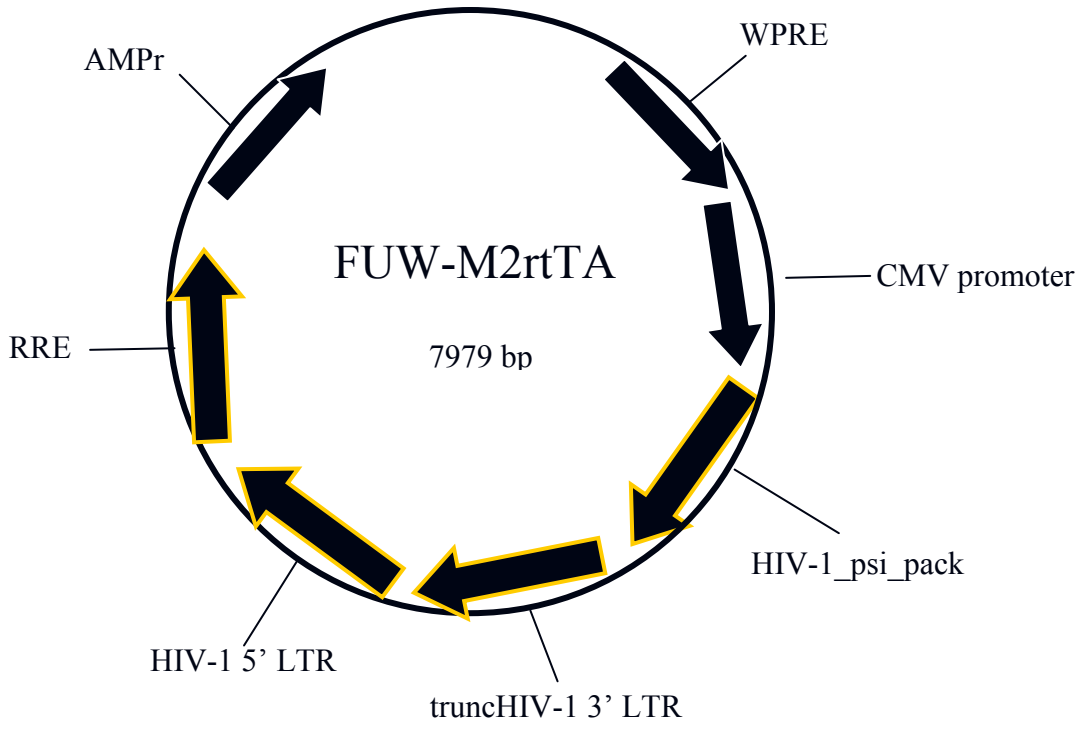


Figure 15. Schematic representation of the FUW-M2rtTA vector.

A

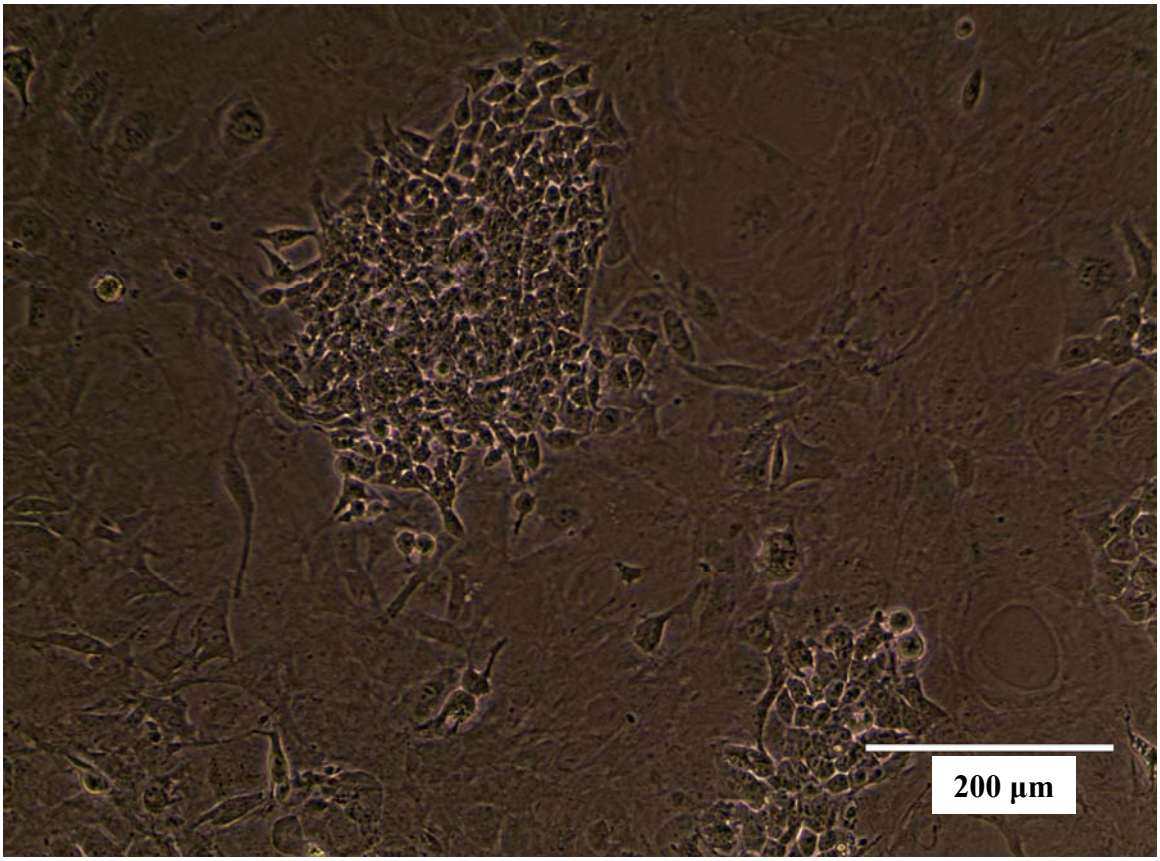


Figure 16. A, B and C, iPS colonies on MEFs coinfected with the pHAGE2-TetOminiCMV-hSTEMCCA and M2rt-TA viruses 4 days postinfection. B, Uninfected MEFs. All pictures are 20x magnification.

B

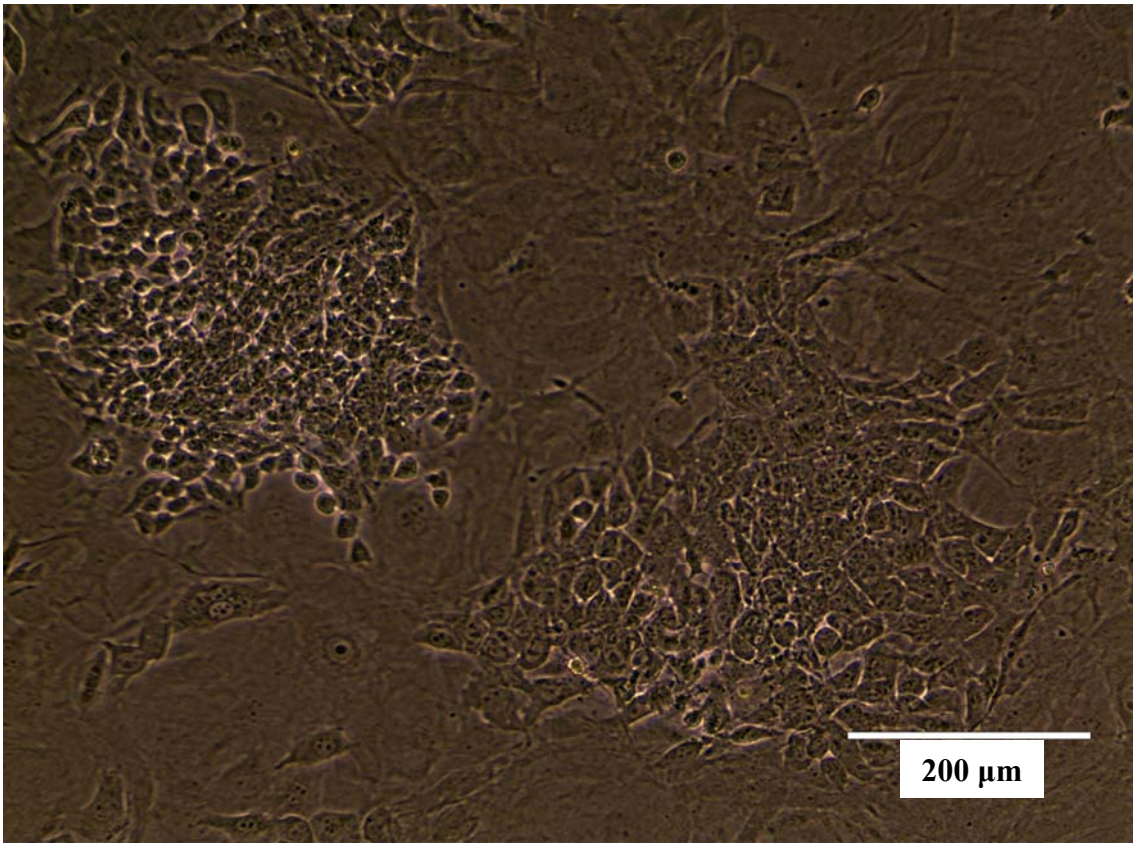


Figure 16, continued.

C

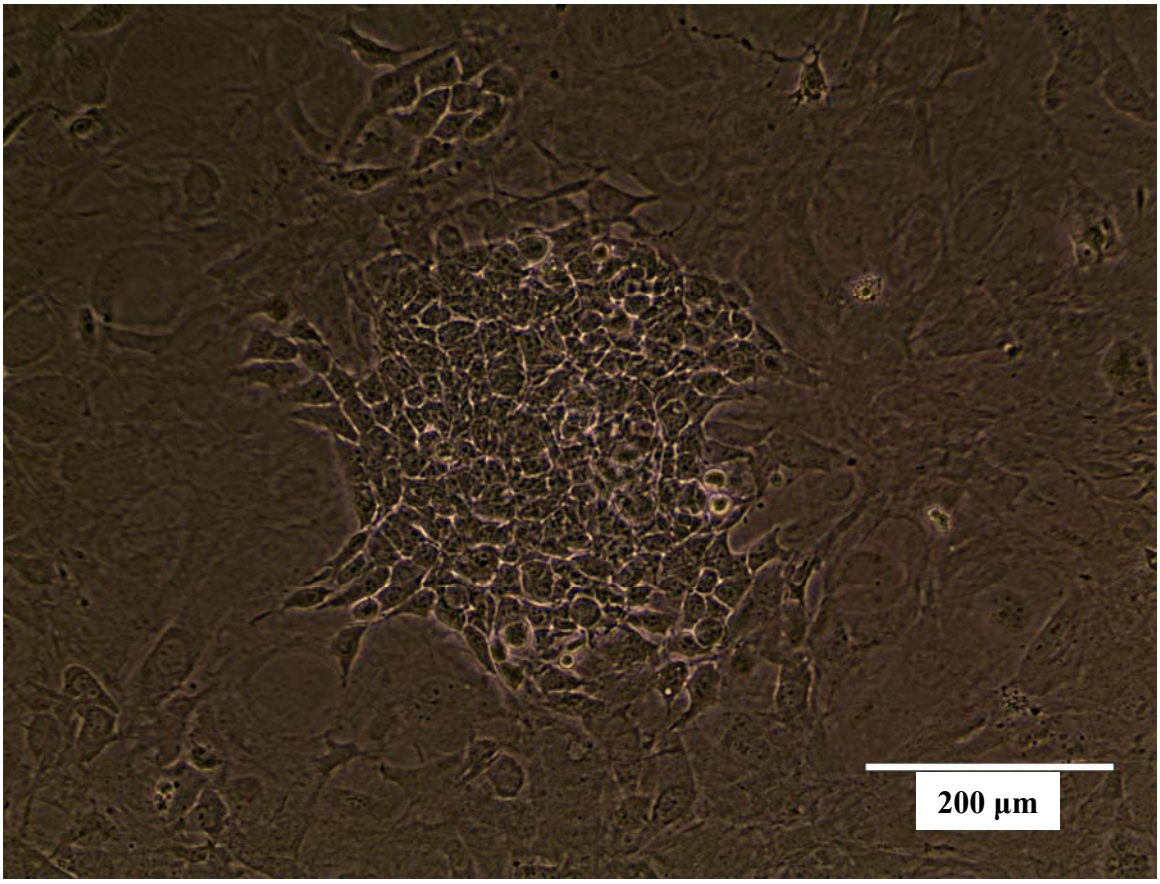


Figure 16, continued.

D

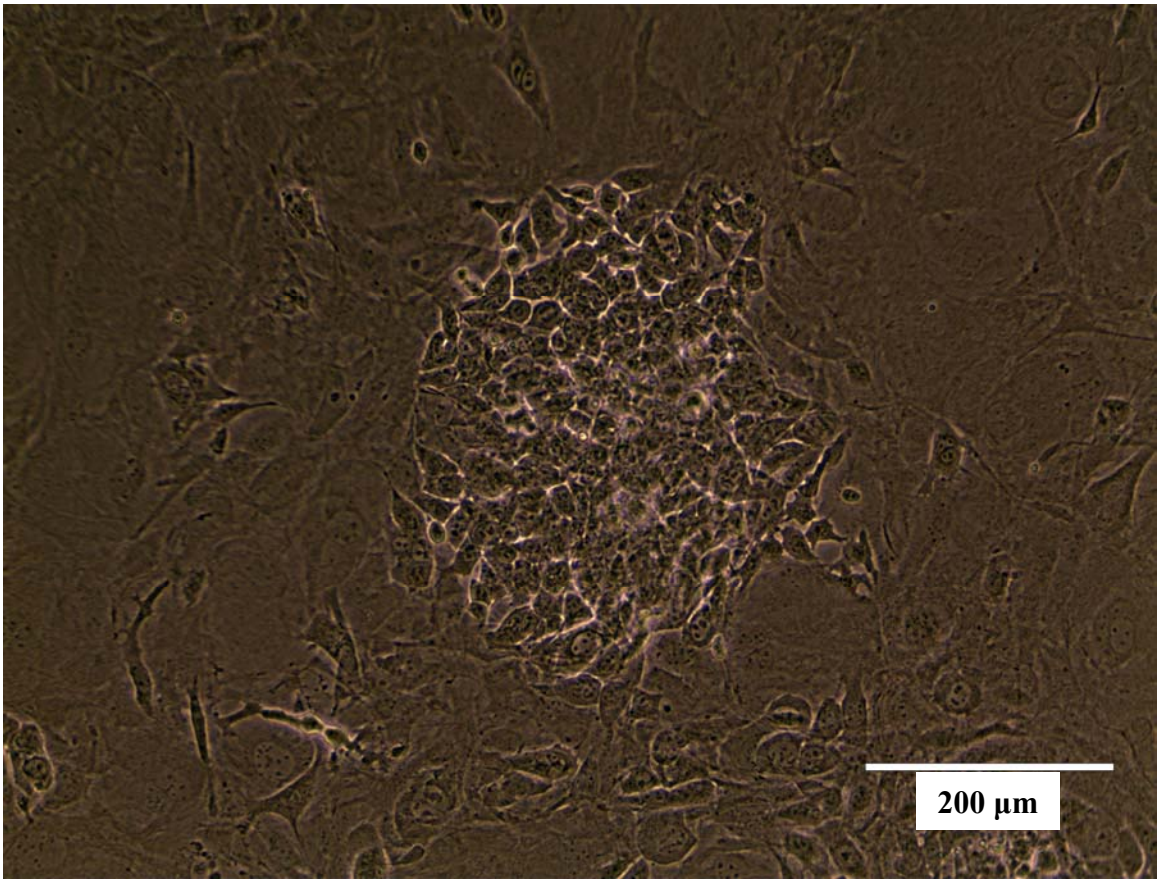


Figure 16, continued.

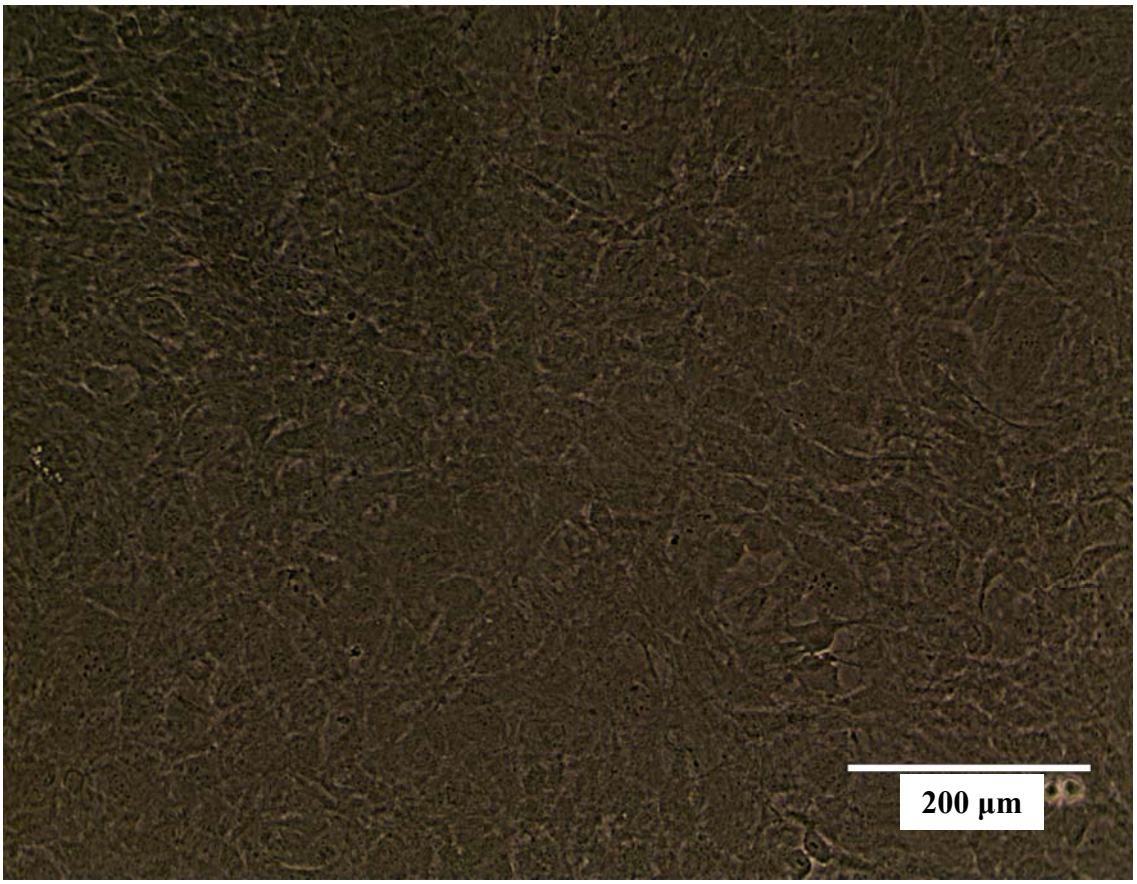


Figure 17. Uninfected MEFs. 20x magnification.

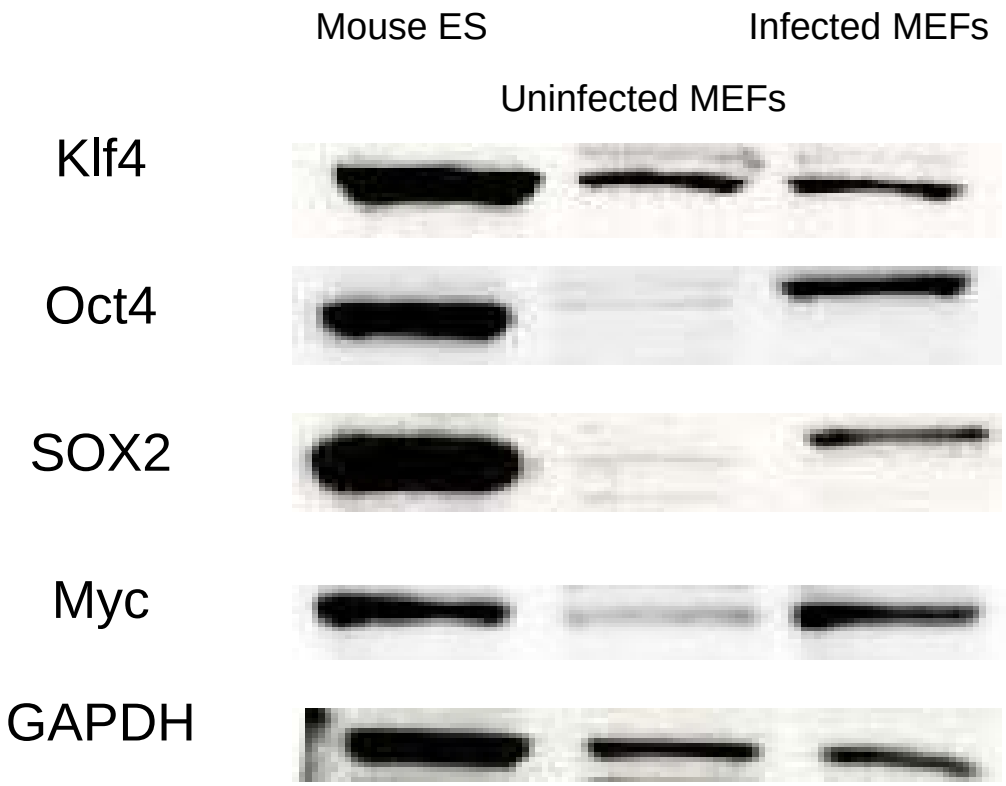


Figure 18. Western blot using protein from mouse ES cells, uninfected MEFs and infected MEFs.

CONCLUSION

Human ES cells are a valuable research tool because they allow us to study the development and function of somatic cells of different types. iPS cells are also very valuable because similar data can be generated without the ethical concerns surrounding the acquisition and subsequent use of ES cells.

The H9 human ES cell line has been acquired and techniques for successfully propagating and cryopreserving these cells have been established in our laboratory. The successful differentiation of ES cells into neurons has been accomplished, and proven with multiple methods. The techniques required to prove that ES cell characteristics are decreased, while neuronal characteristics are increased in these differentiated cells have been optimized and standardized.

The KOSM lentivirus has been characterized, and it has been shown that transcription of the four factors that are necessary for iPS cell formation is not occurring in cells infected with this virus. iPS cells have successfully been created from MEFs using a doxycyline-inducible lentivirus system. Once iPS cells are created from PBMCs, the techniques that have been optimized in this thesis will outline how to drive differentiation into neurons and to characterize these cells.

In conclusion, this thesis provides the necessary framework to study the development and function of neurons from individuals with various neurological disorders.

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